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COLLECTED STUDIES

FROM THE

RESEARCH LABORATORY

DEPARTMENT OF HEALTH

CITY OF NEW YORK

Dr. WILLIAM H. PARK, Director

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In the following pages have been collected all the papers published from the Research Laboratory in the past year, as well as a number of reports and protocols which were unsuited for publication in the regular technical journals. The recipients of this volume will confer a favor on their colleagues of the Research Laboratory by sending their own publications in exchange. Such pamphlets should be addressed to the Librarian, Research Laboratory, Foot of East Sixteenth Street, New York.

New York, September, 1908.

DR. CHARLES BOLDUAN,
Editor.

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THE OPSONIC INDEX AND THE USE OF BACTERIAL VACCINES IN THE TREATMENT OF DISEASE.

William H. Park and Hermann M. Biggs.

Assisted by Drs. Mary E. Goodwin, Charles Bolduan, Marie Grund, Arthur Kendall, and Miss Noble.

Introduction.

Historical—As far back as 1858 Haeckel had observed that particles of indigo injected into the veins of certain molluscs could shortly afterwards be found in the blood cells of the animal. However, the significance of this and other similar observations was not appreciated, until Metchnikoff, in 1883, called attention to their bearing on infection and immunity. The outcome of his investigations was the establishment of the well-known doctrine of *phagocytosis,* the principle of which is that the leucocytes attack and destroy the micro-organisms which have invaded the tissues. Metchnikoff's theory found but little acceptance, the more so since the studies of Nuttall, Pfeiffer, Behring, Ehrlich and others demonstrated a great variety of protective substances in the serum of immune animals. As time went on Metchnikoff realized that the serum played an important part in the destruction of bacteria, but he interpreted this action as being a stimulation of the leucocytes. Denys and Leclef showed that the increased phagocytosis in an immunized animal was due to an alteration in the serum.

In 1903 A. E. Wright, of England, discovered a certain substance in blood serum which united with the bacteria and rendered them more easily taken up by the phagocytic cells. He called this substance *opsonin* and showed that it was present in normal serum and in the serum of infected individuals, though in the latter, as will be discussed directly, it varied considerably from the average normal content. By means of absorption tests, he showed that the opsonin has a specific affinity for the bacteria and none for the leucocytes. Neufeld and Rimpau discovered the same point independently. The opsonins for the staphylococcus prepare only staphylococci, those for tubercle only tubercle bacilli, etc. If fresh, washed leucocytes are mixed with a bacterial emulsion, phagocytosis is very slow, so slow, in fact, that at the end of fifteen to twenty minutes at 37 degrees C. the cells may still be empty. Under the influence of a serum containing opsonin the same leucocytes would have loaded themselves with bacteria, as can be seen by examining such a parallel control. It may be added that the leucocytes are believed by Metchnikoff to be the source of the opsonins. This author shows that the leucocytes act as phagocytes even without free opsonin, only it takes them longer to do it. The work of Hektoen seems to have clearly established that opsonins are distinct antibodies and not identical with bacteriolytic immune bodies. In the further study of these opsonins Wright developed the idea that they were highly important in combating a number of bacterial infections, and soon published observations on the opsonins in tubercle and staphylococcus infections. His results proved to him that inoculations of the corresponding bacteria produced marked changes in the opsonic content of the infected individual, and that it was possible to estimate accurately the immunizing effect of such inoculations. At the present time he has correlated all these observations and has attempted to build up a system of treating bacterial infections by means of active immunization controlled by opsonic measurements.

Technique—Wright's technique of measuring the opsonic power is a slight modification of the Leishman* method and is as follows: An emulsion of fresh human leucocytes is made by dropping twenty drops of blood from a finger prick into 20 c.c. normal salt solution containing one per cent. sodium citrate. The mixture is centrifuged, the supernatant clear fluid removed and the upper layers of the sedimented blood cells transferred by means of a fine pipette to 10 c.c. normal salt solution. After centrifuging this second mixture the supernatant fluid is pipetted off and the remaining suspension used for the opsonic tests. Such a "leucocyte emulsion," of course, still contains an enormous number of red blood cells; the proportion of leucocytes, however, is greater than in the original blood. One volume of this emulsion is mixed with one volume of the bacterial suspension to be tested and with one volume of the serum. This is best accomplished by means of a pipette made by drawing a capillary end to a glass tube. With a wax pencil mark about three-quarters of an inch from the end, it is easy to suck up one such volume of each of the fluids, allowing a tiny air bubble to intervene between the different fluids. All these are now expelled on a slide and thoroughly mixed by drawing back and forth into the pipette. Then the mixture is sucked into the pipette, the end sealed in a flame, and the whole put into the incubator at 37 degrees C. The identical test is made using a normal serum in place of the serum to be tested. Both tubes are allowed to incubate fifteen minutes and then examined by means of smear preparations on slides spread and stained in the usual way. The degree of phagocytosis is then determined in each by counting a consecutive series of fifty leucocytes and finding the average number of bacteria ingested per leucocyte. This number for the serum to be tested is divided by the number obtained with the normal serum and the result regarded as the *opsonic index* of the serum in question. The presence of a high index Wright regards as indicative of increased resistance. He further states that fluctuations of the opsonic index in normal healthy individuals is not more than from 0.8 to 1.2, and that an index below 0.8 is therefore diagnostic of the presence of an infection with the organism tested. It should be stated that for the normal control Wright usually employs a mixture of several normal sera, thus seeking to secure a fair normal average.

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* Leishman, British Medical Journal, January, 1902.

Bacterial Inoculations—Wright's method of treating bacterial infections is based on the following premises: In localized infections the infected body absorbs but small amounts of bacterial substances or antigens. In consequence of this the amount of active immunity developed is but slight. Localized infections therefore tend to run a chronic course. The logical method of effecting a cure in these cases is to actively immunize the body with the invading organism. In a number of infections, notably those of staphylococcus, streptococcus and tubercle, the degree of immunity is measured accurately, acording to Wright, by the opsonic power of the blood serum, *i. e.*, by the opsonic index. He believes it is almost impossible, by mere clinical observation, to determine whether or not the bacterial inoculations are producing increased immunity; that this is comparatively easy by means of opsonic measurements.

The bacterial inoculations consist of suspensions of agar cultures in normal salt solution. These suspensions are heated to 55 degrees or 60 degrees C. for twenty or more minutes to kill the organisms, and then receive small additions of carbolic acid or lysol as preservatives. Cultural tests are made to insure sterility, and the "vaccine" counted so as to determine the dose. This is readily accomplished by means of a small capillary pipette such as is used for the opsonic test. A wax pencil mark is made on the capillary tube and then one such volume of the vaccine to be tested, and one volume of blood taken from a fresh finger prick (an air bubble intervening) are sucked into the pipette. The two fluids are thoroughly mixed by sucking back and forth on a slide and the mixture then spread on a slide in the ordinary way for blood smears. After staining it is comparatively easy to ascertain, by counting through a ruled ocular, the proportion of bacteria to red blood cells. The latter being regarded as 5,000 million per c.c., it needs but a simple calculation to give the number of bacteria per c.c. So far as dosage is concerned one employs from 300 to 500 million in the case of staphylococcus, from 50 to 100 million in the case of streptococcus, etc.

Original Investigations.

Wright, it will have been seen, claims two fundamental points—first, that it is possible to determine the real opsonic power of the blood with sufficient accuracy to make it available for treatment, and second, that the opsonins are either the most important of the protective substances of the blood or that they undergo a sufficient proportional development with the latter to be a safe guide as to their amount.

The Accuracy with Which the Opsonic Power of the Blood can be Determined by Wright's Methods—The opsonic power of the blood is tested by mixing equal quantities of an emulsion of red cells and leucocytes, and an emulsion of bacteria and the serum. This mixture is held at 37 degrees C. for fifteen minutes and is then spread on the surface of a roughened slide. An examination of any slide will show that the different leucocytes vary in their size and in their content of bacteria. This is due partly to variation in phagocytic activity, and partly to the interference of the red blood cells, which are present in great numbers in the emulsion and separate the bacteria in different degrees from the white cells. These and other reasons to be mentioned bring it about that the different leucocytes vary greatly in the number of bacteria they take up and in their distribution on the slide. To overcome this, large numbers of leucocytes are counted. Some enumerate the bacteria in fifty cells, others in one hundred or one hundred and fifty. Beyond one hundred, or at most one hundred and fifty, the increase of accuracy hardly compensates for the extra labor. The following table shows the difference between counting larger or smaller numbers of cells in five opsonic tests as determined by counting different numbers of cells.

Influence of Number of Cells Counted Upon Accuracy.

Cells Counted.	Average Number of Bacteria in each Leucocyte.				
50	1.18	1.88	1.34	1.42	1.60
100	1.22	1.78	1.24	1.42	1.59
150	1.18	1.62	1.22	1.41	1.50
200	1.18	1.51	1.22	1.46	1.37
600	1.28	1.62	1.23	1.39	1.26
1,200	1.34	1.44	1.25	1.30	1.42

It is necessary to have the counts that are compared all counted by the same person, as each individual has a somewhat different method and will average higher or lower for all counts than any other person.

It is noticed that the variation between the average cell count obtained from fifty cells and larger numbers is much greater than between that obtained at one hundred and larger numbers.

When two specimens of blood are tested, not only the inaccuracy of counting due to the different arrangement of the unequally filled cells on the slides to be counted is met, but the fact that in making the test the conditions are not similar, for in different mixtures slightly different proportions of leucocytes, bacteria and red cells will always be mixed together. If smears from a series of tubes of the same blood are compared with a series of smears from one of the tubes, the former will always show the greater variation.

This variation is much greater than most examiners believe. Dr. North collected a series of tests carried out in nearly all the important laboratories in the Eastern United States working upon opsonins. The results recorded seem to us to prove absolutely that while an average counting error of only about 10 per cent. is present, there may be an exceptional error of at least 100 per cent., and one of at least 20 per cent. may be expected once in about every ten determinations.

The following is a fair average of the correctness of routine tests by experienced workers:

[illegible] Count of Bacteria in One Hundred Leucocytes.

Blood Specimen A.		Blood Specimen B.		Blood Specimen C.	
Tube 1	156	Tube 1	142	Tube 1	89
" 2	168	" 2	182	" 2	102
" 3	172	" 3	185	" 3	121
" 4	198				

This error, which occurs because of the technic, applies not only to the examination of the specimen of blood, but also to the measure we employ to estimate the amount of opsonins. As these are not stable, we cannot have a standardized solution, as we do with antitoxins. We must, therefore, determine our measure afresh in each test, taking for this purpose a supposedly normal blood. Wright, from a great many tests, has determined that the opsonic power of the blood in non-infected persons for tubercle bacilli does not vary, as a rule, more than 10 per cent. above or below the average power of healthy blood. For staphylococci there is more variation. It is found also that many things besides infection decrease the amount of opsonins in the blood. Hemorrhage, fatigue, starvation, and other influences which lower the resistance of the body have this effect.

Wright gets this measure as uniform as possible by determining the average opsonic strength of five supposedly healthy persons at the time of each test. If any one of these five is considerably below or above the others it is omitted for that day.

The measure so obtained will probably vary above 5 per cent. from day to day, though seldom getting far away from what we might call the absolute normal. The following results were obtained by us from examining at one test a number of supposedly normal persons against tubercle bacilli and staphylococci.

Opsonic Counts in Test of Twenty-one Normal Sera with the same Stock Staphylococcus Culture.

1	4.13	8	3.82	15	9.09
2	2.93	9	3.95	16	5.17
3	2.78	10	3.98	17	4.04
4	4.37	11	4.27	18	3.82
5	3.58	12	3.69	19	4.00
6	2.90	13	3.80	20	3.79
7	3.56	14	3.59	21	3.44

Opsonic Index in Eighteen Normal Cases with Tubercle Bacilli.

Case.	Average Number Bacilli.	Opsonic Index.
1	2.46	1.00
2	3.20	1.31
3	2.90	1.14
4	2.66	1.08
5	2.75	1.12
6	2.30	.94
7	2.40	.98
8	1.88	.76
9	1.73	.70
10	2.05	.83
11	2.21	.90
12	2.86	1.17
13	2.81	1.14
14	2.79	1.14
15	3.34	1.32
16	2.96	1.02
17	2.16	.88
18	3.12	1.27

The Influence Upon the Opsonic Test of the Specific Differences Between Strains of a Single Species—The general practice in laboratories is to use stock cultures of tubercle bacilli, staphylococci, and other bacteria for the opsonic tests. To obtain a culture from a case may be at first impossible and, if successful, causes a delay of at least one or two days. The culture when obtained may also, as is frequently the case with pneumococci and streptococci, fail to opsonize.

These and other reasons tend to establish the use of laboratory stock cultures, and yet we must acknowledge that when we test the amount of opsonins by both the stock and fresh cultures, a marked difference sometimes develops, so that the index may vary at least 50 per cent. This factor of individual specificity must therefore be taken into account in our interpretation of the accuracy of an opsonic test.

Results from Using Two Different Strains of Staphylococci.

Staphylococcus cultures.

Serum.	First Day's Test.		Second Day's Test.	
	Strain A.	Strain B.	Strain A.	Strain B.
Case 1	8.25	4.09	3.00	2.39
Case 2	6.65	5.92	2.05	1.22

The Leucocytes to be Employed—To many it seems a matter of indifference whether one person's leucocytes or another's is used, but our experiences have shown that the leucocytes from different persons not only vary in their activity, but also in their selective action, and that the index is not the same when obtained with one person's leucocytes as with another's.

TABLE.

Tests of same emulsion of staphylococci with different leucocytes.

Specimen of Blood from	Leucocytes of B. Bacteria per Cell	Leucocytes R. Bacteria per Cell.
Gibson	2.8	2.9
Bolduan	3.0	3.4
Jurist	3.0	3.4
Greenwald	2.6	4.4

The Opsonic Variation During Treatment by Inoculations—Wright lays stress on the considerable uniformity of the degree and persistence of development of opsonins after inoculation. We have found in a small percentage of cases typical increases and decreases, but in the majority of those inoculated there has been great irregularity. Our experience has, of course, been extremely limited when compared to that of Wright.

The following chart for three staphylococcus cases illustrates this:

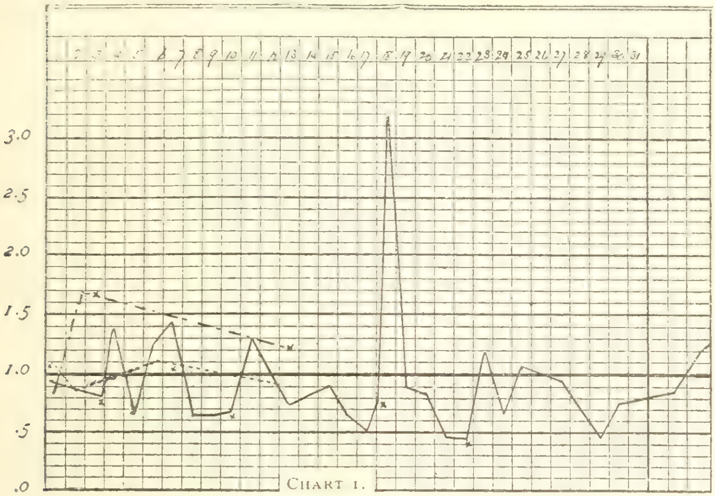
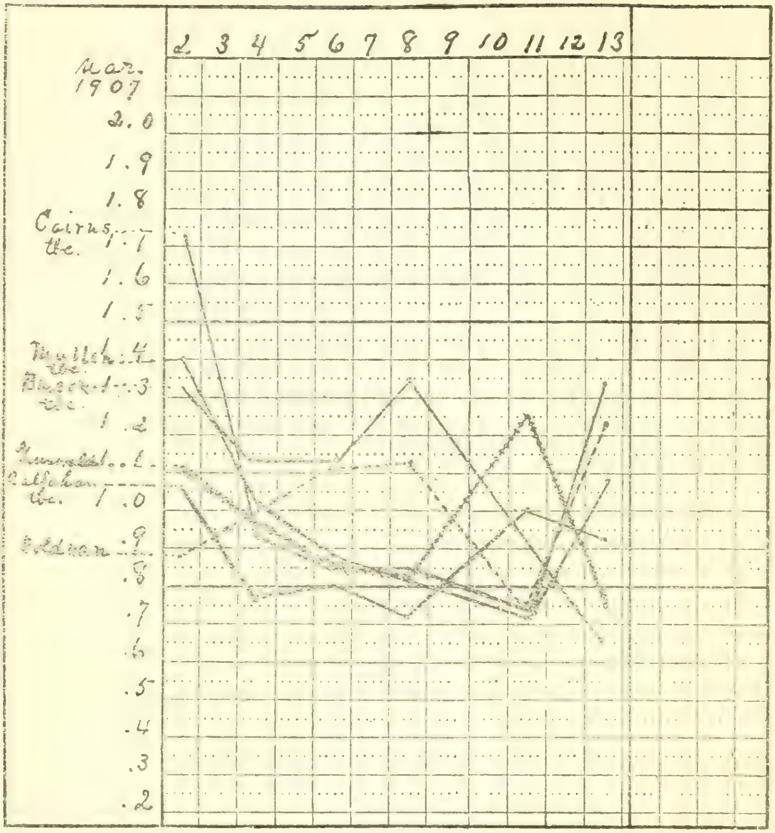


CHART 1.

x = injection of vaccine.

The Variation in the Amount of Opsonins in Supposedly Healthy Persons—It has already been noted that in getting our measure we test a number of persons and exclude the blood of those which vary greatly from the average. The fact that it is necessary to exclude the blood of some shows that variation occurs. We are so in the habit of seeing the normal blood placed at unity because it is each day the measure of comparison that even investigators are apt to think of the indices of normal persons as being unchanged from day to day. This is not the fact. A glance at the next chart, in which three cases of tuberculosis are charted together with two normal persons, shows that the variation is only slightly greater in infected than in normal cases. If one normal person is charted against another for several weeks marked differences will usually appear. The indices of the twenty-one normal cases tested against staphylococci and the eighteen against tubercle bacilli, recorded on page 13, illustrate this variation in the amount of opsonins in normal blood.



Opsonic curves in normal and infected cases.

Dotted lines, normal cases. Continuous lines, tubercle cases (Phthisis).

The Opsonic Index Cannot Be Known at the Time the Treatment Is Given—Most of those who have not carried out inoculations under the guide of the opsonic tests think that the vaccinator is guided at the moment of injection by his knowledge of the opsonic power of the blood at the time. A moment's thought reveals that this is an absolute impossibility. In fact, except under very unusual conditions, it is impossible to have the test of the opsonic power reported within twenty-four hours, and in the treatment of the poor in out-patient practice longer intervals usually elapse, so that the treatment is given on a test made either the day before, or more often on from three to seven days before. As you will see by the three curves on page 15, which are quite as uniform as the average, it is impossible even to guess what the index is at any moment by looking at the indices taken from one to seven days previously. This impossibility of knowing the opsonic power at the moment of inoculation is in itself a very serious difficulty, and should not be overlooked, as it practically is.

The Nature of Opsonins—The work in our laboratories has been, during the past six months, largely devoted to acquiring technic and studying the results of inoculation. Only a little research work has been done on the nature of opsonins.

Wright and Neufeld, in their original experiments, differed as to the effect of heat on opsonins. Wright claimed they were thermo-labile and Neufeld that they were the opposite. Further investigation has shown that the opsonin in those not immunized is largely thermo-labile, and those developed because of immunization are resistant. Muir and Martin believe from their experiments that the thermo-labile opsonin of normal serum and the thermo-stable opsonin are two entirely distinct classes of substances. The thermo-stable substance is of the nature of a true antibody and possesses the comparatively specific qualities of antibodies in general. Powerful complement absorbers have no effect on the thermo-stable opsonin, but do remove almost completely the thermo-labile opsonin.

Emulsions of other than the organisms used in immunization do not absorb a large percentage of the immune opsonin, but do of the complement opsonin.

We have carried out absorption experiments with staphylococci, colon and tubercle bacilli. Our results were similar to those of Muir and Martin. With more closely allied bacteria such as colon and dysentery bacilli, there would be, in all probability, more group absorption.

Comparison Between Opsonins and Bactericidal Substance in the Serum—We have made comparative tests between the opsonic and bactericidal power of the blood in typhoid infection and found that they did not run parallel. The frequent rapid increase in opsonic power within twelve hours of an injection of bacteria is striking and very different from the development of bactericidal strength.

Treatment—The number of cases which we have treated with vaccines has been comparatively few. We began with the treatment of twelve cases of tuberculosis, of which half were tuberculosis of glands or joints and half were incipient lung cases. The opsonic index responded so irregularly to the injections that we were unable to derive help from it in treatment and after four weeks we discontinued taking the index, but continued the vaccine treatment according to the system in use at Saranac Lake and elsewhere.

Nine cases of staphylococcus furunculosis were treated apparently with benefit. In three of the nine, before inoculation were begun, the index was above the normal, in the others it was slightly or considerably below normal. After injection the opsonic power of the blood always rose above the normal, but sometimes only for a few hours. When the index was taken every day, it was so variable as to be of little help, and now we treat the cases without taking the index.

A number of cases of furunculosis came to our attention, during the time when the others were being treated, who refused to be injected. These all made good recoveries, but none of these cases were as severe as several of those receiving inoculation. Our belief is that the vaccines aided in the recoveries of those inoculated.

A number of mixed infections of the sinuses of the head were treated. In these cases great care was taken to thoroughly cleanse the cavities before taking the cultures. Although at first some of these cases seemed to be improved, by giving vaccines prepared from their own cultures, yet in the end only one out of eight showed definite improvement, and the vaccine injections were, therefore, replaced by operation and the usual treatment.

A number of cases of vaginitis in children, due to gonococcus, were inoculated with the gonococcus vaccine. For every case so treated a control was cared for in the ordinary way, and there was no marked difference in the behavior of the two series of cases. A detailed account of these cases is found in the next paper. In a case of gonorrheal arthritis benefit seemed to follow the injections.

Two cases having chronic sinuses leading to diseased bone were treated by the vaccines without benefit. Both were infected with more than one organism. In one of these there were three different organisms, two of these, which were unidentified bacilli, had remarkable indices, one averaging above six and the other above three during several weeks in which the tests were made.

Three cases of acne were treated. Two of these were quite severe and were apparently benefited. They had previously been resistant to the ordinary treatment.

The few cases which we have studied have impressed upon us the opinion that while the vaccines have a restricted field in therapeutics, it is greater than we believed possible before the reports of Wright were published.

Those which appeared to receive the greatest benefit were localized infections of the subcutaneous or deeper tissues. Inflammations of the mucous membranes and generalized infections were not apparently benefited. Our experience in the vaccine treatment is, however, so limited, when compared to that of Wright and his followers, that we feel we are hardly justified in making conclusions even as definite as above.

The opsonic index does not seem to us to be an adequate guide to regulate the use of vaccines.

Other methods of estimating the opsonic power of the blood have been tried, but none of these have seemed to us to be capable of guiding the injections of vaccines in the routine treatment of disease.

VACCINE TREATMENT OF THREE CASES OF GONORRHEAL VAGINITIS IN CHILDREN, WITH A STUDY OF THEIR OPSONIC INDICES.

By Adele Oppenheimer and Harriet L. Wilcox.

Stimulated by the work of Wright and his school, we studied the curative value of gonococcus inoculations in gonorrheal vaginitis in children, and made determinations of the opsonic content of the blood serum during the progress of these cases. We did not, however, use the opsonic index as a criterion for therapeutic purposes, but gave injections at regular intervals of four to eight days irrespective of the opsonic curve. The hospital in which these patients were kept is a considerable distance from the laboratory and not conveniently reached. For this reason daily opsonic examinations were out of the question, and we had to content ourselves with trying to determine the opsonic index, first, immediately before the injection; second, twenty-four hours later, and third, three days later still, and thus follow as best we could the effect produced on the blood serum by the vaccine.

As is well known, in institutions where children are collected together, gonorrheal vaginitis among the female children is relatively common, occurring sporadically or in epidemics, even where, as far as can be discovered, every precaution is taken. For this reason all such cases are rigidly excluded from certain hospitals; but in the public hospitals of a city such exclusion cannot be practiced, and especially not in a hospital for contagious diseases.

In our hospital for contagious diseases careful vaginal smears are made of every female child before admission and where vaginal discharge exists, isolation is enforced. Despite this precaution cases crop out occasionally where the infection has apparently taken place in the hospital. In such instances the child, even when she is well of the disease for which she was admitted, cannot be discharged from the hospital until she is also cured (?) of the vaginitis. Everyone who has studied the question knows that some of these cases of vaginitis are of very long duration; for example, Number One of our series had a profuse vaginal discharge for some five weeks before the vaccine treatment was undertaken, certainly a great additional expense to the city. There was then a practical, as well as a humane and scientific reason for trying Wright's method of vaccination, in the hope of securing a more rapid recovery from the disease than when local treatment alone is given.

One great difficulty which we encountered in the technique was in obtaining a homogeneous emulsion of the gonococcus. The first strain which we attempted to use was very unsatisfactory on account of its clumping. To obviate these clumps, a small amount of sterile sand was added to the emulsions of the organisms, made with different strengths of salt solution, namely, 0.8 per cent., 1 per cent., 1.2 per cent. and 2 per cent., and the mixtures then shaken steadily for five minutes; the sand was thereupon allowed to settle out and the supernatant fluid was examined microscopically, only to find numerous small clumps still present. Different grades of filter paper were then used for filtering a heavy emulsion, but the results were not successful, as the filtrate in each case contained more or less clumps. A second strain of the gonococcus was obtained, which gave a very good emulsion when grown on ascitic agar neutral to phenolphthalein, the growth being much lighter on this medium than on ascitic agar neutral to litmus. Another culture of the gonococcus which gave a very good suspension was isolated by Dr. von Sholly from the vagina of a scarlet fever patient. The child had had a profuse vaginal discharge for four weeks or more, and it was decided to give her vaccine treatment with the autogenous organism and to test the blood for the opsonic content with the same strain.

The technique for measuring the opsonic index used was that devised by Wright. An emulsion of living organisms from a twenty-four-hour culture on ascitic agar was made in sterile 0.8 per cent. NaC1 solution and allowed to stand for an hour or two before using, so that any clumps present might settle out, leaving a good homogeneous suspension. The blood for the leucocytes was collected in sterile 1 per cent. sodium-citrate-salt-solution and centrifugalized for forty-five minutes at medium speed. The normal sera were obtained from three normal adults on the same day as those of the patients and were tested at the same time, and the average phagocytic power of leucocytes under the influence of these three normal sera respectively served as unity. The number of bacteria in each of one hundred polymorphonuclear leucocytes was counted for each serum on each such day, in the belief that this would give us a true indication as to whether the number of organisms in the phagocytes was large or small. The tubes containing the mixture of serum, leucocytes and bacteria were incubated for fifteen minutes and were rotated, one, two, or three times during this period to insure continued mixing of the contents of the tube.

The vaccine was made from a twenty-four-hour culture on ascitic agar, with sterile normal salt solution, and was standardized for one million organisms per cubic centimeter. This emulsion was heated to 65 degrees C. for one hour; cultures were made after half an hour's and after one hour's heating to test the sterility and finally lysol in the proportion of 1-400 was added as a preservative. *After forty-eight hours an abundant growth was found on the ascitic agar inoculated from the emulsion heated to 65 degrees C. for half an hour,* while that inoculated with the emulsion after one hour's heating was sterile.

Nine cases were chosen from which blood was collected and tested. Cases 1, 2 and 3 were children with gonorrheal vaginitis, who received vaccine treatment, together with local treatment part of the time; cases 4, 5 and 6 were also children with gonorrheal vaginitis, but received local treatment only; cases 7, 8 and 9 were normal adults. Case 1 received two inoculations of the vaccine and the blood was tested twice for the opsonic index before it was decided to treat two other children in the same way, and to control these with three unvaccinated children also having gonorrheal vaginitis, *e. g.*, cases 4, 5 and 6. All three cases, 1, 2 and 3, received injections of vaccine made from the organism isolated from case 1, and the serum from each of the nine cases was tested for its opsonic content with this same strain of gonococcus.

On January 3, 1907, a specimen of blood was taken from Case 1 and tested with the autogenous organism; the opsonic index was found to be normal. Two days later the patient was inoculated with one cubic centimeter of the vaccine. On January 7, two days after the first injection, the opsonic index was 1.2 and continued to rise until twenty-four hours after the second injection, when there was a marked fall in the index to .5 below normal. From this time on, the blood was regularly tested, with one exception, on the day it was obtained.

On January 14 Case 2 received the first injection and Case 1 the third. On January 21 Cases 1 and 2 were again inoculated and Case 3 received the first injection. The three cases were then regularly inoculated once a week with one cubic centimeter of vaccine, standardized either for one million or two million organisms per cubic centimeter as shown on the curves.

The blood from these three cases was collected and tested at the same time and under the same conditions as that of the three controls (Cases 4, 5 and 6), and the three normals (Cases 7, 8 and 9). Two of the controls were discharged from the hospital one week after the test was begun, so it was impossible to obtain their blood for further examination, therefore, two other children having gonorrheal vaginitis were substituted as controls.

Histories.

Case 1, *G. J.*—Six and one-half years old. Admitted to Kingston Avenue Hospital on fifth day of scarlet fever. Vaginal smear negative on admission. In her eighth week at the hospital, vaginal discharge noted and smear showed pus cells with gonococcus. Vaginal irrigations with boric acid solution followed by 2 per cent. silver nitrate solution given twice daily. Vaginal discharge persisted in spite of treatment. Vaccination begun about five weeks after discharge was first noted. Improvement after first injection for almost a week; irrigations were stopped when the vaccine treatment was begun, but had to be resumed after two weeks (January 19), on account of profuse discharge. Discharge gradually decreased under vaccine and local treatment, and finally ceased so that the silver nitrate irrigation was stopped (February 4), about one month after vaccine treatment had been started. On February 9 patient was sent home as cured.

Case 2, *G. B.*—Age six. Admitted to Kingston Avenue Hospital with scarlet fever. Vaginal smear on admission was negative. Twelve days after admission, vaginal discharge was noted with pus cells, subsequently gonococci found. Local treatment as in Case 1, except for two weeks after first vaccination (January 14). Vaccination with same organism as in Case 1. Smear was negative February 16 and no vaginal discharge after February 19. Treatment ceased the nineteenth. Patient was discharged on February 24.

Case 3, *S. S.*—Four years old. Admitted to Kingston Avenue Hospital with scarlet fever. Smear was negative on admission. Vaginitis developed five weeks after admission. Received first inoculation on January 21. The discharge gradually diminished. Received local treatment, except for about ten days after first inoculation. Still had vaginal discharge when vaccine treatment was stopped February 19. Local treatment was continued until March 5, when vaginal discharge had completely ceased. Patient sent home on March 12 cured (?).

Cases 4, 5, 6—Diagnosis was scarlet fever. Smears were negative on admission. Vaginal discharge developed one month, two and three weeks respectively, after admission. One received irrigation of boric acid and silver nitrate, two received potassium permanganate irrigations. Two had profuse vaginal discharge with abundant organisms, while blood was being tested. One, Case 6, had very little discharge with occasional organism in pus cells, being practically well, as far as gross discharge was concerned, before we started testing.

Cases 7, 8, 9—Were normal adults, workers in the laboratory. The blood from these cases was collected and tested on the same day as the above with the exception of January 22, when it was not possible to obtain blood from Cases 8 and 6.

Conclusions.

Our work has been carried on upon so few cases that we can give but tentative conclusions.

(1) Every one of the six patients, both those with and those without vaccine treatment, was discharged from the hospital as cured within twelve weeks from the beginning of the gonorrheal vaginal discharge. The patient who suffered longest from the gonorrheal vaginitis (Case 3), had both forms of treatment for a while, but, after all, she was only ill of gonorrheal vaginitis six weeks after the first and two weeks after the last inoculation. Moreover, Case 4, with only local treatment, showed a discharge for about ten weeks. Of the other two patients who received both kinds of treatment, and the injection as well as the irrigation to the last, Case 1 was cured (?) in about four weeks and Case 2 in about five weeks after the first injection.

On the other hand Cases 5 and 6, who were given local irrigation only, were cured (?) in about five and one-half weeks and in about three weeks respectively.

In comparing these very few cases of gonorrheal vaginitis in children, namely, the three with vaccine treatment, in addition to local treatment, and the three with only irrigation treatment, we see that the vaccine treatment did not shorten the duration of the disease markedly, if at all.

(2) Nothing in the general or local conditions of the cases explained the opsonic indices on the various days on which they were estimated, but

(3) There were greater fluctuations in the opsonic curves of the vaginitis cases than in the normal, especially above unity rather than below. The apparent exception in Case 6 will be made clear by reading the history of the case, which shows Case 6 to have been practically normal most of the time.

(4) After each inoculation, the opsonic index apparently rose or continued to rise, excepting only in Case 3, between January 28 and February 4.

(5) We did not observe any negative phase (which, according to Wright, immediately follows a bacterial inoculation), following the injection, but it should be remembered that the blood was not tested until twenty-four hours after the inoculation. In this experience, we agree, however, with those in our laboratory who have worked with microorganisms other than the gonococcus, and who tested the opsonic power shortly after inoculation with bacterial vaccines. (See the preceding paper by Park and Biggs).

(6) Judging by the curves of the three cases of vaginitis which were not vaccinated, auto-inoculation is hard to avoid, and therefore the opsonic curve does not promise to be of value in suggesting the times for injection.

(7) Judging by the opsonic curves of the normal cases, what Wright would call a low opsonic index is not of diagnostic value; on the other hand a comparative study of the curves leads one to suspect that an unusually high index might serve as a diagnostic hint.

(8) Finally, by the way, our results with normal sera and gonococcus confirm those fluctuations reported by Bolduan* of the Research

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* Bolduan, Long Island Med. Journal, Vol. I., 1907.

Laboratory with regard to normal sera and tubercle, staphylococcus and streptococcus, namely:

(a) The opsonic power often differs very widely in different normal individuals. See curves of Cases VII., VIII. and IX.

(b) From day to day the fluctuations of the opsonic index of one and the same normal serum are great and that without any apparent cause. See curves of Cases VII., VIII. and IX.

(c) There is an enormous difference in the phagocytic activity of the different leucocytes under the influence of the same serum in the same tube, as exhibited by the difference in the number of ingested bacteria. For instance, on one slide, taken at random, the average number of gonococci in a polymorphonuclear leucocyte was 2.86; each of ten polymorphonuclear leucocytes contained 10 or more gonococci, and each of forty-three such leucocytes contained none at all.

We wish to thank Dr. Binford Throne, of the Kingston Avenue Hospital, for his generous assistance there.

Supplement.

After the work described above was completed, we had the opportunity to study comparatively, but for less than a month only, three additional cases of gonorrheal vaginitis in children, namely, Case 10, given vaccine treatment only, Case 11, given irrigation treatment only, and Case 12, left untreated.

The method pursued was exactly like that described above, except that in obtaining the leucocytes, we centrifugalized, not forty-five minutes at moderate speed, but fifteen minutes at high speed. In our previous investigation we had discovered that such a difference in the manner of centrifugalization made no difference whatsoever in the leucocytes. On most days the normal sera were obtained from one normal adult, and from two children convalescent from scarlet fever.

The vaccine was made from the organism obtained from Case 10 and standardized for two million organisms per cubic centimeter. All the sera were tested for the opsonic content with the organism isolated from Case 1. The serum from the vaccinated patient and the two control sera (from the irrigated and untreated cases respectively), and the normal sera were collected at one time under the same conditions.

On March 21 and 28 and on April 4 Case 10 was inoculated with the autogenous organism. On each of these days before the injection and each time on the day following the injection, a specimen of blood was taken from Case 10 and was then tested for the opsonic index. Also two or three days before the inoculation the serum was collected for testing.

On these self-same days (namely, on March 19, 21 and 22*, 25, 28 and 29, and on April 1, 4 and 5), blood was collected for estimating opsonic content from the control Cases 11 and 12 and from four normal Cases 13 and 17, and 15 and 16.

In addition, between March 8 and April 19 the opsonic indices of Cases 10 and 11 (vaccinated and irrigated respectively), and of the normal Cases 13, 14, 15, 17 were obtained a few times as indicated in the curves. The normals were changed when, for one reason or another, it was impossible to use their blood for further examination.

Histories.

All the cases, excepting only Case 13, were children admitted to the Willard Parker Hospital with scarlet fever.

Case 10, *R. G.*—Three years and four months old. Gonorrheal vaginitis on admission. Vaccine treatment was begun nineteen days after admission and given until this investigation was discontinued, that is, the vaccine treatment was given for about two weeks.

Case 11, *F. P.*—Age four years. Gonorrheal vaginitis on admission. Vaginal irrigations with potassium permanganate given twice daily throughout her stay in the hospital. While her discharge was somewhat less, her smears still showed gonococci when she left the hospital.

Case 12, *H. M.*—Six years old. Gonorrheal vaginitis on admission. Gonococci still present up to the time that we stopped taking her blood.

Case 13—Normal adult, worker in the laboratory. The blood from this case was collected and tested, as normal to gonococcus, on the same days as the above with the exception of March 8 and 11, when it had not yet been decided to use this blood.

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* Cases 11 and 12 not examined on March 22d.

Case 14, *J. M.*—Girl, age three years and two months. The blood from this case was collected as normal to gonococcus, on March 8 and 11.

Case 15, *L. F.*—Girl of four years. The blood from this case was collected and tested, as normal, from March 8 to 25, inclusive.

Case 16, *W. D.*—Boy, age four years. The blood collected, as normal, from March 28 to April 5, inclusive.

Case 17, *S. T.*—Boy, four years old. The blood collected, as normal, from March 15 to April 5, inclusive.

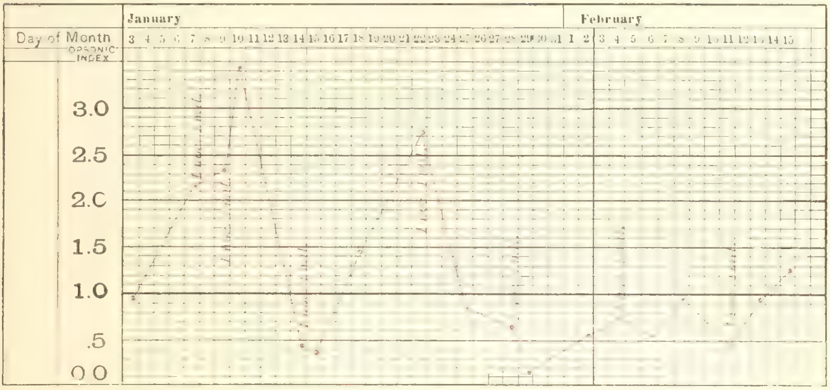
Summary.

(1) No one of the three patients had been cured of gonorrheal vaginitis up to the time when the investigation had to be discontinued, so no further light was thrown on the value of the vaccine treatment.

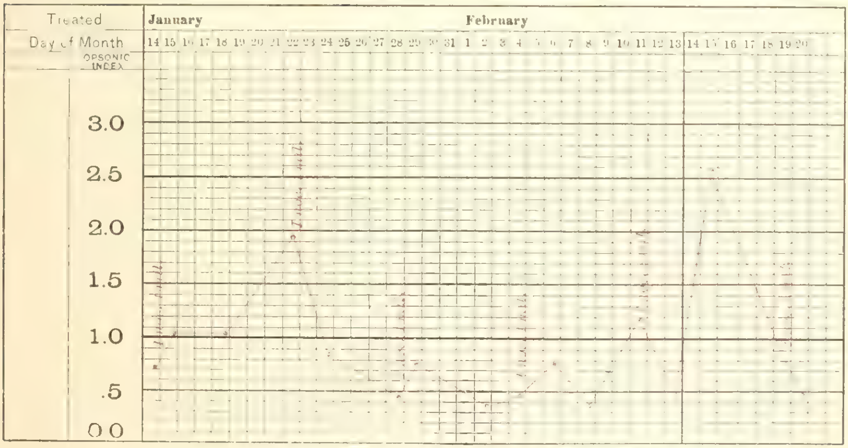
(2) All the other conclusions of this study, namely (2) to (8), inclusive, on page 25, were corroborated by this supplementary work.

OPSONIC INDEX CHART

NAME *Goda Judelson* **DIAGNOSIS** *Vaginitis* **CASE NO** *I*

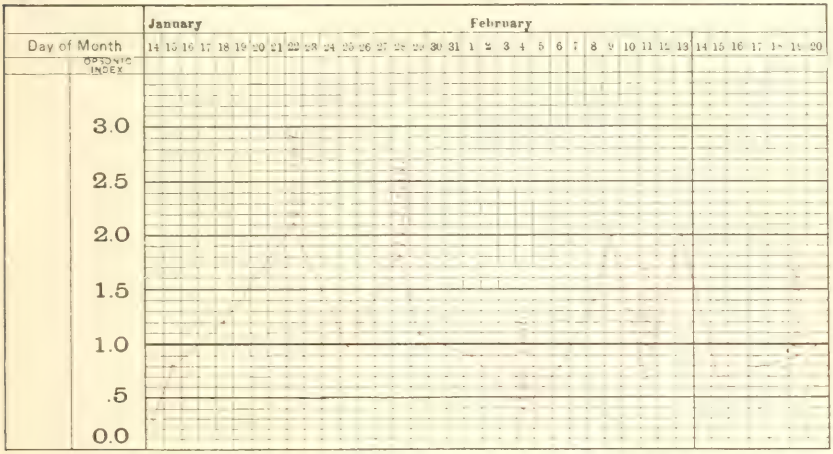


NAME *Gussie Berman* **DIAGNOSIS** *Vaginitis* **CASE NO.** *II 2*

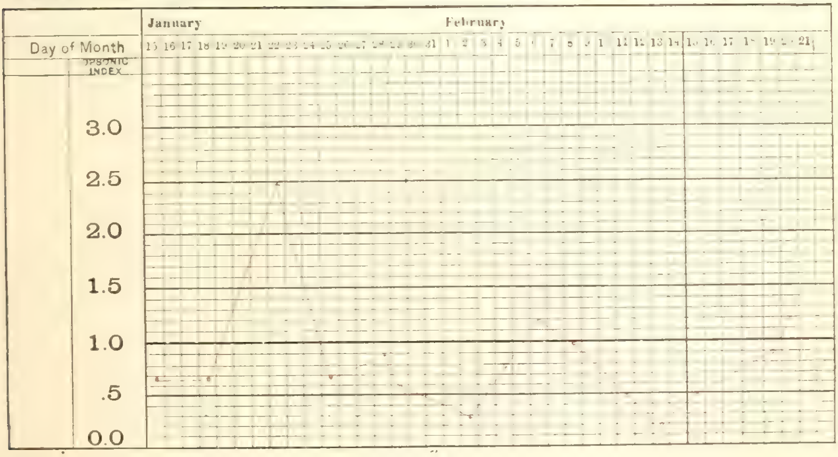


OPSONIC INDEX CHART

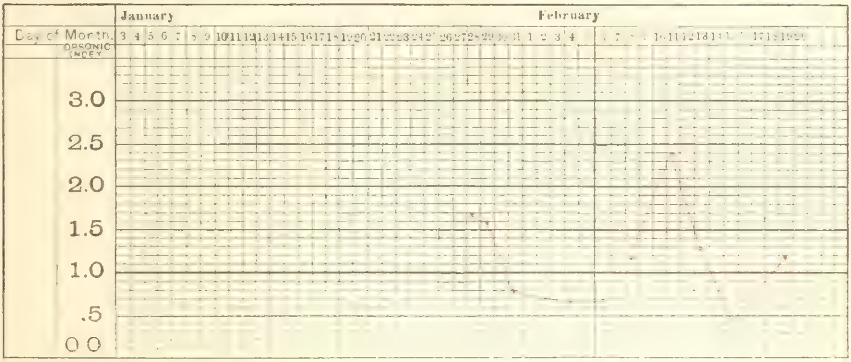
NAME *Sutherland, Sadie* DIAGNOSIS *Vaginitis* CASE NO. *III*



NAME *Giuseppe Tarasco* DIAGNOSIS *Vaginitis* CASE NO. *IV*

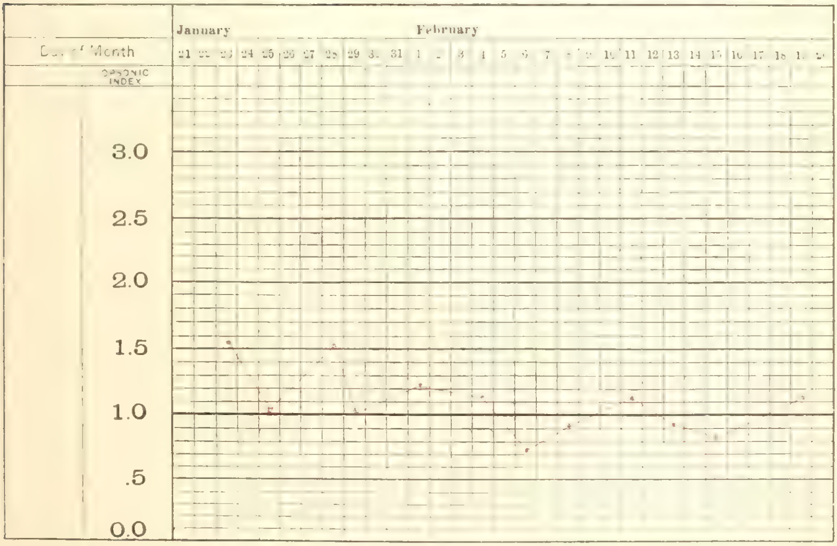


NAME Moretsky, Sadie DIAGNOSIS Vaginitis CASE NO. V

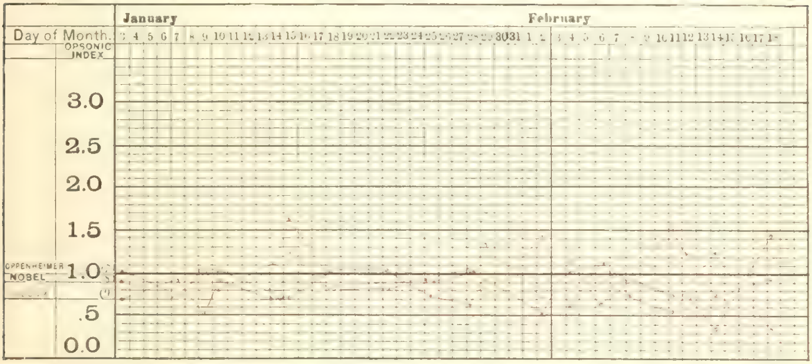


OPSONIC INDEX CHART

NAME Oxenhandler, Mollie DIAGNOSIS Vaginitis CASE NO. VI



NAME *Normals ÷ by av. of 3 Normals* **DIAGNOSIS** .... **CASE NO.** *VII VIII IX*



THE IMPORTANCE OF ICE IN THE PRODUCTION OF TYPHOID FEVER.*

William H. Park, M. D.

We may endeavor to settle this question directly by determining whether epidemics or scattered cases of typhoid fever have been traced to ice, or, failing in this, we may try to estimate the probability of such infection by learning the duration of life of the typhoid bacillus after freezing.

The total number of instances of typhoid fever which have been directly traced to ice infection are remarkably few. I have been unable to trace more than three. One was in France, where a group of officers placed ice made from water polluted by a sewer in their wine and afterward developed typhoid fever, while those of the same company not using ice escaped. A second case was in a small epidemic brought to my notice which occurred in those who used ice from a pond. It was found that water directly infected with typhoid feces had flowed over its frozen surface had been congealed there. A third case followed the use of ice from the St. Lawrence river, gathered at a point at which sewage pollution was marked. In this instance bacilli were isolated from the ice which were believed to be typhoid. If typhoid fever is communicated through ice, except under exceptional conditions, it is remarkable that so few cases are traced to it.

The fact that freezing kills a large percentage of typhoid bacilli makes it indeed possible to conceive that ice from moderately infected water contains so few living typhoid bacilli that only the exceptional person here and there becomes infected, and so the source of the infection remains undetected.

If this be true and scattered cases occur, there should be at least some increase on some or every year in March, April and May in such a city as New York, where four-fifths of all the ice consumed is from the Hudson River, which is known to be contaminated with typhoid bacilli. The people of New York use ice very freely and most of them put it directly in their water or place their vegetables on it. The new ice from the Hudson River is gathered in January or February and stored on top of the left-over ice, and thus shipments to the city are immediately begun. It is an established fact that typhoid bacilli in ice are most abundant during the days immediately after freezing. At the end of two months less than 1 per cent. of the original number survive.

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*Read in the Section on Hygiene and Sanitary Science of the American Medical Association, at the Fifty-eighth Annual Session, held at Atlantic City, June, 1907.

If Hudson River ice produced an appreciable amount of typhoid fever, this would then be noticeable in March and in April and perhaps in May.

When we examine the records for the past ten years we find no increase of typhoid fever in Greater New York during those months, with the one exception of the present year, when we had in the Borough of Manhattan a sharp outbreak lasting four weeks. This outbreak did not occur at all in Brooklyn. As the people of Brooklyn drank different water, but received ice from the same places of the Hudson River as those of Manhattan, this directed attention to the water or milk rather than the ice. Examination of the Croton watershed at the time showed that a small epidemic of typhoid existed there and that pollution of the water was probable. This suggested still more strongly that the water and not the ice was the cause of the typhoid infection.

It happened that most of the cases occurred in those living in the section of the upper West Side, where only well-to-do people live. An investigation showed that the majority of the infected had used only artificial ice and several had used no ice in their water at all.

Let us now turn our attention to the life of the typhoid bacillus in ice in laboratory experiments. The first important investigation was that of Prudden, who showed that typhoid bacilli might live for three months or longer in ice. This experiment is frequently wrongly interpreted, as when a recent writer states: "It has been amply demonstrated that the germs of typhoid fever are not killed by freezing and that they have been known to live in ice for long periods of time."

It is true that in Dr. Prudden's experiment a few typhoid bacilli remained alive for three months, when the experiment was terminated, but these were but a small fraction of 1 per cent. of the original number. Following Prudden's experiement, Sedgwick and Winslow in Boston, and I in New York City carried on independently a series of experiments. These led to the same conclusions. A table summarizing a final experiment of mine in which twenty-one different strains, mostly of recent isolation, were subjected to the test is given below:

Life of Twenty-one Strains of Typhoid Bacilli in Ice.

	Average Number of Bacilli in Ice.	Percentage Typhoid Bacilli Living.
Before freezing	2,560,410	100
Frozen three days	1,089.470	42
Frozen seven days	361,136	14
Frozen fourteen days	203.300	8
Frozen twenty-one days	10.280	0.4
Frozen twenty-eight days	4.540	0.17
Frozen five weeks	2.950	0.1
Frozen seven weeks	2,302	0.09
Frozen nine weeks	127	0.005
Frozen sixteen weeks	107	0.004
Frozen twenty-two weeks	0	0

In these experiments twenty-one different flasks of Croton water were inoculated each with a different strain of typhoid bacilli. In one a little of the feces rich in typhoid was directly added. The infected water in each flask was then pipetted into thirty tubes. These tubes were placed in a cold storage room in which the temperature varied from 20 to 28 degrees F. At first tubes were removed and tested twice a week, later once a week. The object of using so many different strains was because it has become evident that some cultures lived longer than others.

At the end of five weeks the water infected with six cultures was sterile, at the end of sixteen weeks only four strains remained alive.

Two interesting investigations of Hudson River ice have just been completed, one by Dr. D. D. Jackson, for the city, and the other by Dr. C. E. North, for a private laboratory.

There was noticed a considerable difference between the number of bacteria in the top, middle and bottom layers of ice. This is natural, since while water in freezing from above downward markedly purifies itself, 75 per cent. of the solids and a fair proportion of bacteria being eliminated, yet this can not happen in the case of the snow blanket which becomes flooded by rain or by cutting holes through the ice. Here all impurities, such as dust and leaves which have fallen on the surface and dirt which may come from the water, remain with the bacteria which they carry. The bottom ice, being the most recently frozen, has had least destruction by freezing. Dr. Jackson's report gives us a rather unpleasant impression of Hudson River ice. He states that most of the samples either at the top or bottom show intestinal germs. The worst portions of the ice showed from 1 to 10 bacilli of the colon group per c.c. These were taken above or less than five miles below Albany. Some of this ice might aptly be termed solidified sewage.

The analysis of the twenty-five samples shows, as one would expect, that the surface of the ice just at Albany was badly contaminated, one c.c. of melted ice averaging about 15,000 bacteria for four samples. Only one of the twenty-one samples taken at different distances below Albany showed over 1,000 per c.c. A sample from top of a cake of ice obtained two miles below Albany showed only 14. The other twenty samples had on the average 105 per c.c.

Dr. North, in his investigation, examined the ice from forty spots between Hudson and Albany. He took samples from the top, middle and bottom of each cake and the water of the river. He has kindly permitted me to use some of his figures.

The river water in the forty specimens averaged 1,800 bacteria per c.c., the top ice 306, the bottom ice 36 and the middle ice 14. Only four specimens of top ice had over 500 bacteria per c.c., none of the specimens of middle or bottom ice.

Thirty-three of the specimens of water had over 500, and 23 over 1,000. Colon bacilli were obtained from but one specimen of the middle ice, two from the bottom ice and twelve from the top ice.

The great destruction by freezing is noticeable in these figures. Even the top ice soiled by the horses and men gathering it contained but 16 per cent. as many bacteria as the water from which it was contained. The bottom ice, the last to be frozen, had but 2 per cent. of those in the water.

Conclusions.

The danger from the use of ice produced from polluted water is always much less than the use of the water itself. Every week that the ice is stored the danger becomes less, so that at four weeks it has become as much purified from typhoid bacilli as if subjected to sand filtration. At the end of four months the danger becomes almost negligible, and at the end of six months quite so. The slight danger from freshly cut ice, as well as the natural desire not to put even sterilized frozen sewage in our water, suggests agreement with the report of Dr. Jackson, that portions of rivers greatly contaminated, such as the Hudson within three miles of Albany, should be condemned for harvesting ice for domestic purposes—such ice alone to be used where there is absolutely no contact with food.

STUDIES ON THE ETIOLOGY OF SCARLET FEVER.

By Drs. Anna Williams and May Murray Lowden,
Assisted by
Dr. B. V. H. Anthony and Miss C. Gurley.

Studies on scarlet fever have been carried on intermittently at the Research Laboratory of the Health Department for the last five years.

Our opportunities for the study of this second most common acute infectious disease have steadily increased in the past few years, and they are now exceptional. With an immense scarlet fever hospital at our door, full of patients, with an expert hospital staff ready to assist us in every way, practically the only drawbacks in our work are, first, the inability to obtain autopsies when death occurs during the active stages of the disease, and, second, the fact that so far as we know, man is the only animal susceptible to the disease. We are, therefore, unable to test our findings on experimental animals. The experiments on monkeys have as yet been too few for us to draw any conclusions as to their complete non-susceptibility.

Historical Review.

Notwithstanding the fact that this disease has been recognized as an entity since the time of Sydenham (1685), and that there is a lengthy bibliography in regard to it, we really possess comparatively little knowledge of its exact nature. This is due in great measure to the lack of careful observations and of minute records. Even from a clinical standpoint we are still in doubt in regard to many points.

(1) In the first place, we do not know definitely the distribution of the virus, and hence the chief source of infection.

Prior to 1893 the desquamating skin is most frequently mentioned as carrying the contagion of scarlet fever, though it must be noted that as early as 1883 Pohl Pinkus insisted on the disinfection of the mouth. During the nineties, the French authorities especially laid stress on the infectivity of the secretions of the mucous membrane of the throat, nose and ear, ignoring the desquamating skin. Since then, most writers have laid equal stress on both as possible modes of infection. In 1896 discharges from post scarlatinal otorrhoæ are mentioned as infectious, and hospital records bear witness to the truth of this observation. Later, urine, faeces, perspiration and even the breath, are spoken of. Since 1905 the general opinion seems to be that the primary desquamation and the secretions from the mucous membranes of the nasal and buccal cavities carry infection, but that the prolonged desquamation probably does not.

The great majority of so-called "return cases,"* according to recent statistics are thought to be due to infective material contained in the throat, nose and ear exudates. In order to be certain of this, the most careful oversight of discharged hospital cases should be carried on for some time after their return home, since a fresh attack of exudative inflammation in an apparently cleared-up case of recent scarlet fever is supposed to start up the development of remnants of quiescent scarlet fever virus.

Our discharged cases are now being kept sight of, as far as possible, with the idea of solving this problem.

(2) We do not know how long the virus may remain infective, when separated from the patient. The most extravagant tales are told of infection from exposed articles after twenty years' rest in a trunk.

(3) We do not know the exact period of incubation in this disease. The majority of authors think that the first symptoms may show any time from twenty-four hours to four weeks after exposure, while some recent good observers say that in all cases recorded as developing under four days, the possibility of an earlier exposure cannot be ruled out, and that the mean time of the different observers, twelve to fourteen days approximately, is probably the true period of incubation in this disease.

According to the work done on experimental infectivity, there seems to be but little doubt that the period of incubation may be as short as twenty-four hours. For instance, it has been shown that children can be infected with scarlet fever within twenty-four hours after inoculations subcutaneously with the mucus from the throat or mouth of scarlet fever patient.

In 1897, J. W. Stickler, of Newark, claimed to have produced genuine scarlet fever in ten children by such inoculations. The mucus was first treated with carbolic acid, 1/600th part, before inoculation. All the children developed the scarlet fever symptoms beginning in less than twenty-four hours.

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* By "return cases" we mean cases which in all probability have become infected from discharged hospital cases.

Leube (Rep. Archives of Pediatrics, October, 1905), injured his finger during an autopsy on a severe scarlet fever case. On the seventh day the wound pained, on the tenth day there was angina and malaise and later a rash appeared.

(4) It has been shown by this experimental work on infectivity that it is difficult to produce scarlet fever in animals. The inoculations by which Stickler produced scarlet fever in children, in white rabbits gave only local effects. Grünbaum, in 1904, was successful in producing a scarlatinal tonsillitis in a chimpanzee by rubbing the throat of the chimpanzee with swab from the patient's throat. On the fourth day a doubtful rash developed; on the fifth day an exudate on the tonsils appeared, which soon covered both tonsils. The sixth day the rash spread mostly over the abdomen but was not scarlatiniform in appearance. "Streptococcus conglomeratus" was isolated from the throat of the chimpanzee. The monkey's own blood agglutinated it. Grünbaum tried many other inoculation experiments. He found the streptococcus isolated from scarlet fever cases fatal to rabbits, but producing only suppuration in monkeys.

A disease resembling scarlet fever has at times been reported in cows. In Great Britain from 1882 to 1892, inclusive, seventeen epidemics (varying from 5 to 635 cases), were traced directly to a disease in the cows supplying the families infected. In eight of the cases mention is made of an eruption on the udder. In 1885, during the epidemic at Marylebone, due to disease in the Hendon cows, Klein isolated a streptococcus from these animals, and after comparing it with streptococci isolated by him from scarlet fever cases (1886-7), he believed the organisms were identical and the disease was scarlet fever.

Dr. Park investigated an epidemic of scarlet fever at Ossining several years ago, which occurred in the practice of Dr. Barnum, and considered that it was undoubtedly caused by the use of milk from a septic cow. The cow had recently calved, had a high temperature and great quantities of streptococci were found in the milk and in the vaginal discharge. There were over thirty cases of scarlet fever in the two boys' schools which received the milk. The cases showed symptoms forty-eight hours after drinking the milk. None of the day scholars, who did not drink milk, developed the disease, and there was no scarlet fever in the town. Dr. Park reports that while the individual cases appeared as mild scarlet fever, the outbreak on the whole seemed somewhat different from ordinary scarlet fever. There was only one case which contracted scarlet fever from these boys.

(5) Though much has been written about the rôle of the streptococcus in scarlet fever, and, while many still hold that the streptococcus pyogenes, or a closely related streptococcus, is the real cause, there is no positive proof that this is so. The descriptions given of the organisms isolated and studied are too indefinite to allow satisfactory corroboration, and, therefore, claims for a specific variety of streptococcus amount to little. It is impossible to draw any conclusions from the reports of the earliest writers, because of their inadequate knowledge of bacteriologic technic. The first clear description of a streptococcus isolated from scarlet fever was given by Klein in 1886. He isolated the organism from the blood of five out of eleven early cases of the disease. He lays stress upon the irregular morphology and conglomerate growth (in broth), in distinguishing from other streptococci.

Baginsky, Summerfield, Ruediger, Jochmann, etc., found "streptococcus" in the blood of living cases of scarlet fever. Among the workers who have continued the study of the subject, the following have expressed the belief that the streptococcus is the cause of scarlet fever—Berge, Sverenson, Brunner, H. Fischer, Baginsky, Summerfield, D'Espine, Moser, Marignac, Kurth, Gordon and Gabritschewsky—but the growing opinion seems to be that the streptococcus, though closely associated with scarlet fever is a secondary invader of the system and not the cause. Hektoen, Jochman, Besredka, Dopter, Mallory, Duval and Siegel express this opinion, while Ruediger, Cumpston and others are non-committal.

Jochmann (in 1904), examined the blood of 161 living cases of scarlet fever and found "streptococci" in 25 (about 15 per cent.). Out of 66 autopsy cases 50 showed the streptococcus in the blood (over 80 per cent.).

Of the recent workers, Gabritschewsky speaks most positively in favor of streptococcus as the cause of scarlet fever, stating that his experiments in the use of streptococcus vaccine furnish new proof in favor of the streptococcus origin of this disease. In one series of 120 children vaccinated with streptococcus emulsion, he states that 13 per cent. showed symptoms of scarlet fever.

In regard to the variety of streptococcus isolated from scarlet fever by the various authors there is much diversity of opinion. Many simply speak of isolating "the streptococcus." Some consider that the streptococcus of scarlet fever is identical with streptococcus pyogenes.

Class, in 1889, isolated a diplococcus which he considered the specific cause of scarlet fever, but no other investigators agree with him in this.

In 1891 Kurth described the "streptococcus conglomeratus" as the cause of scarlet fever. Klein states that Kurth's description of this streptococcus agrees with his own, and that, therefore, the two organisms are probably the same species. Gordon also described a similar streptococcus.

Baginsky, 1900, states that he found the same streptococcus in the blood and bone marrow of 42 scarlet fever autopsies, but that it could not be distinguished from other varieties of streptococcus.

We cannot find any mention, in Gabritschewsky's articles of 1906 and 1907, of the variety of streptococcus used for the vaccine emulsions more than "streptococcus" from a scarlet fever patient; nor does he mention the culture generations used for the inoculation.

Marmorek recognizes no varieties of streptococcus. Besredka and Dopter (1904), state that no cultural or microscopic appearances or pathogenic actions seem to differentiate the streptococci. Agglutination is useless. They hoped for some help from the Bordet-Gengou reaction, but their hopes were not fulfilled. Foix and Mallein (1907), on the contrary, report positive results on using this method.

Moser says that the differences so far given between the streptococcus of scarlet fever and other streptococci are not sufficient to differentiate them, so other methods of differentiation must be sought. The streptococcus Moser used for some of his experiments showed short chains and diffuse cloudiness in bouillon.

Baginsky describes diplococci or short chains in the blood smears, long chains in the bouillon cultures, which returned to short chains after passage through animals.

From the above short resumé of the bibliography we see how impossible it is to arrive at a positive opinion in regard to a specific streptococcus in scarlet fever. The report of Anthony, working in this laboratory, on the hemolyzing qualities of streptococci in scarlet fever is given in another part of this volume.

(6) Only one of the many clinical symptoms of scarlet fever is said to be pathognomonic.

The *rash* is not characteristic, for we may obtain a perfectly typical scarlet eruption after inoculation of antitoxic or normal serum. *Desquamation* may occur after any erythema. Similar *throat symptoms* appear in ordinary tonsilitis.

The one symptom said by some to be pathognomonic is the enlargement of the papillae at the tip and sides of the tongue, and even this is sometimes so slight that it is difficult to make out, and it has been reported as occurring in other diseases.

The *complex of symptoms* is said to be charateristic in the majority of cases, but still many cases occur on the border line between serum rashes, measles and scarlet, and there has been much discussion over the "Fourth Disease of Duke," without arriving at a definite conclusion.

(7) Very little has been done on the minute pathology of scarlet fever, due largely to the reasons already given, that deaths occur so seldom during the height of the disease, and that it is so difficult to obtain autopsies. The only constant general change found is a hyperplasia of lymphatic tissue over the whole body.

Most attention has been given to the skin in the histologic study of this disease. Hebra and Kaposi describe hyperaemia. Klein was the first to make a careful study of the skin and there is no other report until that of Pearce in 1899.

He studied the changes in the skin on the different days of the disease, finding the infiltration with polymorphonuclear leucocytes most marked between the fifth and eighth days. He found the same epithelial changes in the pharynx, palate, and tonsil. In the tongue the leucocytes were most marked in the epithelium covering the papillae.

In 1904 Mallory studied the skin in living and autopsy cases, and described the bodies which he believes to be protozoa and the cause of scarlet fever, finding them in four autopsy cases. Two of these were of the severe toxic type and two were septic scarlet fever complicated with diphtheria. Rash was present in all four cases. Mallory found the skin from a number of living cases as well as from many autopsy cases negative. In his four cases he found the bodies in three situations, lying in vacuoles in the epithelial cells of the epidermis, to a less extent between these cells, and free in the lymph channels of the corium just beneath the epidermis. They were found usually in small clumps. The bodies were of two types, granular, or reticular, and radiate. Mallory gave the name "cyclasterion scarlatinale" to these bodies because of the radiate forms which he considered the more characteristic. Mallory's work has been little corroborated up to the present time.

In a study published in 1905, Duval agrees with Mallory that the bodies are protozoa and the cause of scarlet fever. He examined the serum from blisters, formed by ammonia, in 18 cases, and found the "Mallory bodies" in 5. He says also that he found them post-mortem in the skin of an infant whose blister serum showed them during life.

Field (1905), in our laboratory examined the skin from 20 living scarlet fever patients and from 10 autopsies, as well as from a number of controls, and came to the conclusion that most, if not all, of the bodies were degenerations of the cytoplasm of the host cells. He found none in living skin.

In 1905 R. L. Thompson, of St. Louis, states that he believes the Mallory bodies "to be protozoa and to have an etiological significance to scarlet fever," but that their significance cannot be made clear until they can be studied in cultivation.

In 1906 Leonard S. Dudgeon failed to find the bodies in sections from two cases which died on the fourth day with well-developed rash. He describes his technic as follows: Pieces of skin from a fatal case of toxic scarlet fever in an adult male. Death on fourth day of disease. Skin was fixed in Zenker and in salt formalin and stained with Leishman's stain (using a 0.5 per cent. solution of Leishman's powder in methyl alcohol).

Five of the recent editions of bacteriologies and pathologies mention the Mallory bodies in considering the possible cause of scarlet fever. McCollom in Osler's Modern Medicine (1907), says "the cause of scarlet fever is probably a protozoan as Mallory's work seems to demonstrate."

Several other workers have described protozoa-like bodies in scarlet fever, but their findings have not been confirmed, and are only added because of their historic interest.

Doehle in 1891 reported two forms of parasites found in the blood of five scarlet fever cases, one small flagellum-bearing organism, the other larger and granular.

In 1893 Pfeiffer states that he found a flagellum-bearing, freely motile amoeba in the blood serum of scarlet fever as late as the third day after the appearance of the eruption, and also found the same organism in variola, vaccine, zoster and measles.

Behla in 1893 describes small round protoplasm bodies, at times with flagella, in the blood of measles and scarlet fever.

J. Siegel in 1905 describes bodies in the skin and blood of scarlet fever, which he also found in small-pox, syphilis and foot and mouth diseases. He could not differentiate them in the various diseases, except in the skin by their position. His bodies are not like the Mallory bodies.

(8) Finally, more knowledge in regard to the geographic distribution of scarlet fever may be of assistance in studying its etiology.

The disease is very common in Germany, England and United States; common over the greater part of Europe (Italy one-half as many cases proportionately as England), Canada and South America; uncommon in Asia and Africa and in a few islands. It is rare in India, Madagascar and Japan. As late as 1903 two authorities pronounced it unknown in Japan. Ten cases were reported from Tokio Hospital in 1898 by M. J. Mayeda. (See Pediatrics, 1898, Vol. VI.).

McCollom, in Osler's Modern Medicine, mentions a zone of comparative immunity through part of the torrid zone. In comparing the geographical distribution of scarlet fever with erysipelas and puerperal fever as types of streptococcus diseases, we find the distribution of the two latter more general than that of scarlet fever, but in the countries where they are most prevalent scarlet fever is also prevalent, and in India and Japan where they are rare, scarlet fever is rare.

Original Work—Having noted one or two small points which we considered advantageous in histologic technic while working on rabies, we decided to examine another series of scarlet fever cases, following, to a certain extent, the rabies technic in preparing our specimens.

Methods—Our technic was as follows: Very small pieces were fixed for a short time in Zenker's fluid. The important point here is the length of time in the fixative; from 5 to 10 hours we found best. From the Zenker fluid they were passed successively through iodine-alcohol, 70 per cent. and 96 per cent. alcohols, each for 24 hours, then for two hours in two absolute alcohols, cleared in cedar oil, imbedded in paraffin, and stained by various methods, chief among them being the following: Mallory's, iron-hematoxylin, safranin and "Lichtgrun," Unna's polychrom, Giemsa, safranin and gentian violet.

Specimens from two cases were prepared according to Levaditi's process for demonstrating spirochetes, and specimens from three cases were fixed in Fleming's solution and submitted to various stains.

Altogether sections of skin from 17 living cases, from 33 autopsies, and from 9 controls were examined. The following is a list of the cases examined, with some indication of the findings:

Table I.

Living Cases.

Case No.	Age.	Days Ill when Specimen was taken.	Intensity of Rash in General.	Characteristics of Rash at Time Specimen was Taken.	Microscopical Exam.	
					Bodies in Lymph Spaces.	Bodies in Epithelial Cells.
41	27	4	±	General erythema	+	—
42	2⅓	3	±	Light erythemation on neck, abdomen and chest	+	—
44	5	*21	+	Typical scarlet erythema	—	—
59	2	3	+	Punctate—macular	±	—
60	21	7	+	Slightly faded. Primary desquamation on neck	±	—
61	21	6	+	Macular, beginning desquamation of neck and arms	+	+
62	16	4	+	Macular, well-developed	+	±
63	9	3	+	Macular	±	—
64	6	3	+	Macular	±	—
65	5	4	+	Slightly macular	—	±
66	5	3	+	Macular	—	±
70	4	6	+	General erythema	—	—
71	8	4	+	Macular and punctate, beginning vesicular	±	—
72	11	5	++	General intense erythema, beginning macular eryp. Desquamation on back	—	—
73	3	3	+	General punctate	—	+
74	6	5	+	General macular; beginning desquamation on neck	—	±
75	27	3	+	Light erythema slightly faded	+	±

* Case of recurrent scarlet fever with typical rash 21 days after first symptoms and rash which were typical. Desquamation occurred after each attack. Blood culture was negative.

TABLE II.

Autopsy Cases.

Case No.	Age.	Days Ill.	Intensity of Rash.	Appearance of Skin Post Mortem over Areas Cut for Sections.	Complications.	Sections Hours, P. M.	Culture.	Microscopical Examination.	
								Bodies in Lymph Spaces.	Bodies in Epithelial Cells.
1	4½	3	[illegible]	....	Broncho-pneumonia; diphtheria following measles.	8½	....	+	+
2	2½	22	±	....	Diphtheria, mastoiditis....	33	....		+
11	[illegible]	2 (?)	+	Chest, desquamating; abdomen, macular; thigh, macular and desquamating....	Diphtheria................	4	....	++	++
12	8	22	+	Thigh, desquamating; back, P. M. redness........	Vaginitis, conjunctivitis; broncho-pneumonia; meningitis...............	4	....	—	—
13	3	4 (?)	+	Lower thigh, hemorrhagic spot; back, macular.........................	Cervical adenitis..........	6	Blood, + Pus, +	+	+
50	[illegible]	7	+	Back, P. M., redness.................	Broncho-pneumonia........	7	Blood, +	±	±
53	5	6	[illegible]	....	Cervical adenitis; myocarditis......................	Same day.	Blood, + Pus, +	±	
200	3½	5	++ hemorrhagic	Ankle, hemorrhagic spot; wrist, spot; abdomen, eruption......	....	14	Blood, +	++	++
217	3½	4	[illegible]	Side, P. M., redness, eruption; thigh, clear; back, P. M., redness, chest, eruption....................	....	5½	—	+	+
S.C. 24	2	13	++ hemorrhagic spots.	....	Broncho-pneumonia, pericarditis; otitis media....	10	Blood, + Pus, + Blood, +	+	+
25	6	8	(?)	Back, P. M., redness.....	....	11	—	+	+
30	27	8	+	Chest, P. M., redness; chest desquamating	Nephritis, dermatitis, herpetiformis...............	23	Blood, +	++	++
34	6	(?)	Desquamating.	Desquamating......................	Erysipelas, nephritis, cancrum oris................	9	....	—	—
37	21	(?)	(?)	?	?	2	Blood, —	+	—
39	2½	6	+	....	Diphtheria.................	2	Blood, —	—	—

43	6	4	+	....	?	a 10 to 15 min. b 24 hrs.	—	++	++
48	1	6	+	Back, P. M., redness..................	Vaginitis, conjunctivitis....	2½	+	±	—
49	2	13	+	Marked desquamating and possible rash	Broncho-pneumonia........	3	—	±	±
53	7	5	+	Back, P. M., redness............... ...	Diphtheria, meningitis....	18	....	+	±
56	1½	2	+	At time of death, general scarlet.....	Mastoiditis, broncho pneumonia..................	29 hrs. 20 min.	....	+	±
57	9	4	(?)	....	Septicemia, diphtheria......	4½	....	+	±
58	1½	..	..	Back, P. M., redness............... ...	....	6½	....	++	—
67	3	10	+	No rash visible.........................	Diphtheria	2	....	-	
68	12	7	+	Vesicular	Diphtheria	8½	....		—
69	5	7	+	....	Broncho pneumonia........	15 min.	....	±	—
92	6½	3	+	....	Enlarged cervical glands, myocarditis.............	4	Bl. No. gr. Pus. +	—	—
94	8	5	+	....	....	35 min.	Bl. No. gr. Pus.........	+	—

Table III.

Control Cases.

Case No.	Age.	Days Ill.	Intensity of Rash.	Characteristics of Rash.	Complications.	Sections, hours, p. m.	Microscopical Examination.	
							Bodies in lymph space.	Bodies in epith. cells.
Measles, 31 living	10 months.	1	+	General macular, papular eruption, beginning to fade	Broncho pneumonia.	....	±	+
Measles, 32 living	5 years.	1	+	General macular, papular, little faded on chest	....	....	—	—
Measles, 33 living	2 years.	1	+	Macular, papular eruption on extremities	....	....	—	—
Measles, 34 living	3 years.	2	+	Macular, papular eruption on face	....	....	±	—
Measles, 35 living	8 months.	2	+	Macular, papular eruption, beginning to fade.	....	....	—	—
Measles, 36 living	2 years.	3	+	Diffuse bright rash, fading on chest	....	....	+	—
550, autopsy	....	..	..	Deep burn	Septicaemia.	....	++	++
Diphtheria, autopsy, 307	14 weeks.	7	..	Anti toxin rash, scarlatina in form, persisting almost up to death	Broncho pneumonia intubation	15 mins. 18 hours 25 hours	—	—
Smallpox, autopsy, 207.	57 years.	5	++	Vesicular eruption over entire body; purulent on face, confluent on hands and legs	Slight systolic mitral murmur. Epileptic convulsions.	48 hours	..	..

In one of the living cases we found scattered groups of small reticular bodies in the lymph spaces, and in six of these an occasional similar small body within an epithelial cell.

In 27 of the autopsy cases, bodies have been found, in the lymph spaces in 20, in the epithelial cells in 15. In the majority of these, many bodies are seen, but they are practically all of the granular or reticular type. In several of the autopsy cases a few interesting groups of these finely reticular bodies were observed on the lymph spaces just beneath the epithelium. The forms are so characteristically amoeboid in outline and are so definitely grouped that they are quite suggestive of amoeboid protozoal forms. Through the kindness of Dr. Mallory, we have been able to study a number of sections from one of his cases, and we find that the reticular amoeboid bodies seen in our cases are quite similar to amoeboid forms in his sections. We found, however, no definite radiate or star-shaped bodies in any of our cases, and only occasionally did we see an indefinite rosette, while in the sections from Dr. Mallory's case both the rosettes and star-shaped bodies are very numerous and very definite. If these are specific organisms it seems strange that we have found comparative so few forms, and these the less characteristic ones, in all of our cases. It might, however, be accounted for by the fact that certain organisms (some protozoa), show only characteristic forms for a short time at definite stages of their life history, and produce tiny indefinite forms throughout the greater part of their existence. This is notably the case among certain amebida. One of us has an ameba under observation now which was isolated from the feces of a cat suffering from dysentery. This ameba in the adult form is a very small organism, with a definite nucleus. It changes quickly, however, by multiple budding into many tiny amebae, only a few of which show, with the best of staining, merely traces of nuclear material. The great majority of them appear as tiny reticular amoeboid forms showing no nuclei, very similar to the great majority of the forms found in scarlet fever. The finding, therefore, only of small apparently non-nuclear, reticular bodies, should not be considered strong evidence against such forms being protozoa.

On the other hand it is true that similar small reticular bodies may be formed as the result of degenerative changes in the tissues. We found in one of our control cases many bodies apparently identical with the reticular bodies found in our scarlet fever skins.

This control skin was obtained from an autopsy case after death from extensive burn. The section of skin in which the bodies were most frequent is taken from the margin of the burn. The skin constains streptococci (strept. pyogenes), and small bacilli (B. vulgaris). This one control would throw out the majority of Mallory forms as being protozoa, unless two things were true; first, the child may have had scarlet fever; but this can practically be ruled out because no definite history of scarlet fever was obtained and no cases of infection followed. Second, the technic may not have brought out definitely what really existed, that is, the recticular bodies from the burn might show quite a different structure from those found in scarlet fever, with a different or yet undiscovered technic. In this case there were many transition stages from undoubted cytoplasmic degenerations to the definite reticular bodies, which is a strong point in favor of the cytoplasmic nature of all the bodies.

Among the control cases we examined pieces of skin from six living measles patients. In sections from these cases we have found focal areas of leucocytic infiltration of the intercellular spaces of the epithelium similar to those seen in the scarlet fever skins and occasionally we have seen a few small indefinite reticular bodies in the lymph spaces and in the epithelial cells, much more indefinite than most of those found in scarlet fever. In one case a very definite small reticular body with a central granule was seen in an epithelial cell, the reticulum staining a clear blue, the central granule a little deeper blue, with eosin and methylene blue staining; but of course little or nothing can be inferred from one form.

More characteristic of these measles skins is the presence of certain *hyaline* amoeboid forms found in the lymph spaces of the corium, especially beneath the areas of leucocytic infiltration of the epithelium. These forms have been seen in all the measles skins, but not in large numbers; they vary in size from that of a large coccus to that of a red blood cell. We have been unable to detect any definite structure in them, therefore they do not correspond with the small nucleated forms seen by Field in measles. The forms described by Field we have not seen in the skin from the six living cases studied by us.

Besides these irregular hyaline bodies we have seen on the margins of the areas of leucocytic infiltration of the epithelium, lying between the epithelial cells, small collections of tiny delicate granules arranged in irregular groups and short rows. They are not definitely enough grouped or distinctly enough stained to be considered organisms, but they are in sufficient numbers to bear closer study. The measles cases have not yet been studied minutely enough to warrant a separate report. As controls for the scarlet fever skins, we can say that none of them show characteristic Mallory bodies.

In all of the sections of skin from the scarlet fever cases examined by us the reticular bodies found were in small definite areas. So it is possible that we may have missed some foci, but since many serial sections were examined in most of the cases we can say at least that the foci, if present, must be widely scattered. It is interesting to note that in one autopsy case a piece of skin taken immediately after death shows as many bodies as one taken twenty-four hours later. By referring to the table it will be seen that we found no relationship between the number of bodies and the presence of streptococci in the blood.

So far, then, in our study of these bodies, we have found no definite evidence of their being organisms; neither have we been able to show that they are merely degenerative tissue changes. It seems to us, however, that the evidence is in favor of this latter view of their nature.

Furthermore, we have been unable to demonstrate anything like a tiny organism within the larger bodies, therefore, we cannot agree with Prowazek in including scarlet fever in his group of diseases produced by what he calls Chlamydozoa. Prowazek's studies were made principally on vaccinia and trachoma. He has reported no studies on scarlet fever or rabies, and yet he includes both these diseases in his group. It seems to us that he has generalized from too few particulars.

No spirochetal forms were demonstrated by the Levaditi method.

Most of our work, up to the present time, has been on scarlet fever skins, but we feel now that more promising results may be obtained from a minute histologic examination of the exudates and of the superficial tissues of mouth and nose, and possibly of the lymphatic system in general. We are, therefore, planning work along these lines for the immediate future.

Bibliography.

Baginsky, A.—Uber Antistreptokokkenserum bei Scharlach. Berlin klin. Wochenschr., 1902, Nos. 48, 49, pp. 1113, 1152.

Baginsky, A., & Summerfield, P.—Uber einen constanten Bacterienbefund bei Scharlach, Berlin klin. Wochenschr., 1900, No. 27, 28, pp. 588, 618.

Banks, A. G.—Variations in scarlet fever of the opsonic power of streptococci. Journ. of Path. and Bact. 1907, XII., 113.

Besredka & Dopter—Contribution a l'étude du role des streptococques au cours de la scarlatine. Ann. de l'Institut Pasteur, 1904, XVIII., 373.

Buerger, L.—Differentiation of Streptococci by Fermentative Tests. Journ. Exp. Med. 1907, IX., 428.

Class, W. J.—The diplococcus scarlatinae. Illinois Med. Journ., 1903-4, V., 77.

Cumpston, H.—Relative frequency of various types of streptococci in scarlatina. Journ. of Hyg. 1907, VIII., 599.

Dohle—Zur Aetiologie d. Masern, Pocken und Scharlach. Centralb. für Bact., 1895, XII., 909.

Duval, C. W.—Die Protozoen des Scharlachfiebers. Virchow's Archiv. f. path. Anat., 1905, CLXXIX., 485.

Field, C. W.—On the presence of certain bodies in the skin and blister fluid from Scarlet Fever and Measles. The Journ. of Exp. Med. 1905, VII., 343. See also, Collected studies from the Research Laboratory, Dep't of Health, New York City, 1905, I., 19.

Foix & Mallein—La Reaction de Bordet et Gengou vis à vis des Streptocoque dans la Scarlatine. Press Mèd. 1907, XV., 777.

Fraenkel—Ueber Menschenpathogene Streptokokken. Munch. Med. Wochenschr., 1905, XII., 548, and XXXIX., 1868.

Gabritchewsky, G.—Ueber Streptokokken Vaccine und deren Verwendung bei der Drüse der Pferde und dem Scharlach des Menschen. Centralb. Bact. Orig., 1906, XLI., 719 and 844.

Gabritchewsky, G.—Uber Streptococcen Erytheme und ihre Beziehungen zum Scharlach. Berl. klin. Wochenschr. 1907, XVIII., 556.

Gordon, M. H.—Further report on the bacteriology of scarlatina. Report of Local Government Board of London, 1901. Also see Lancet, 1905, 2, p. 1400. "A ready method for differentiating strep tococci."

Grünbaum, A.—Some experiments on enterica, scarlet fever and measles in the chimpanzee. Brit. Med. Journ. 1904, I., 817.

Harris, Norman MacCleod—The relative importance of streptococci and leucocytes in milk. Journ. of Infect. Dis., 1907, Supplement No. 3, p. 50.

Hektoen, L.—Bacteriological examination of blood during life in scarlet fever with special reference to streptococcemia. Journ. Am. Med. Ass. 1903, XL., 685.

Hektoen, L.—Is scarlet fever a streptococcus disease? Journ. Am. Med. Ass. 1907, XLVIII.

Jamieson, W. A., & Edington, A.—Observations on a method of prophylaxis and an investigation into the nature of the contagium of scarlet fever. Brit. Med. Journ. 1887, I., 1262.

Jochmann, N. G.—Bakteriologische Blutuntersuchungen an 161 Scharlachkranken (intra vitam). Deutsches Arch. f. klin. med., 1903, LXXVIII., 209.

Jochmann, N. G.—Bakteriologische und Anatomische Studien bei Scharlach, mit besonderer Berücksichtigung der Bluntunterschung. Mitt. a. d. Hamb. Staatskrankenanstalt, 1904, IV., 120.

Jochmann, N. G.—Bakterien Befunde bei Scharlach und ihre Bedeutung für den Krankheits-process. Zeitschr. f. klin. Med. 1904-5. LVI., 316.

Klein, E.—Annual Reports of the Local Government Board of London (supplements containing the Report of the Medical Officer):

1885-6, Disease of cows at Hendon farm, p. 90 (See also W. H. Powers' report in same journ. p. 73).

1886-7, Etiology of scarlet fever, p. 367.

1887-8, Experiments on cows with streptococcus scarlatina, p. 203.

1896-7, Microbes associated with scarlatina, p. 267.

Kurth, H.—Uber die Unterscheidung der Streptokokken, und über das Vorokmmen derselben insbesonderer des Strept. conglomeratus bei Scharlach. Arbeit a. d. Kaiserl. Gesundheitsamte, 1891, VII., 389.

Mallory, F. B.—Scarlet Fever; protozoan-like bodies found in four cases. Journ. Med. Research, 1903-4, X., 483.

Mandelbaum, M.—Zur Streptokokken Frage. Zeitschr. f. Hyg. 1907, LVIII., 26.

Mayeda, M. Y.—About Scarlet Fever in Japan. Pediatrics, 1898, VI., 544.

Menschikoff, W. K.—Zur Serum-therapie des Scharlachs. Berl. klin. Woch., 1905, 1223; also see, Wien. klin. Woch. 1905, p. 1223.

Meyer, F.—Bakteriämie bei Scharlach. Deutsche med. Wochenschr. 1906, XXXI., 2033.

Moser, P.—Ueber die Behandlung des Scharlach mit einem Scharlach-streptococcenserum. Wiener klin. Woch., 1902, XLI., 1053.

Nedrigailow, W.—Zur Frage uber die spezifische Natur des Scharlach streptococcus. Centralb. f. Bakt. 1906, XLII.,13.

Pearce, R. M.—Scarlet Fever, its bacteriology, gross and minute anatomy. Journ. Boston Soc. m. Sc., 1898-9, III., 161.

Reudiger, E. H.—The bacteria in scarlatinal and normal throats. Journ. Am. Med. Ass. 1906, XLVII., 1171.

Schick, B.—Uber die weiteren Erfolge der Serum-Behandlung des Scharlach. Deutsche Ded. Woch., 1905, XXXI., 2092.

Schottmüller, H.—Zur Aetiologie der Pneumonia crouposa. Münch. Med. Woch., 1905, LII., 1425.

Schultze, W. H.—Zür Differentialdiagnose der menschenpathogenen Streptokokken. Münch. Med. Wochenschr. 1907, XXIV.

Siegel, John—Unterschungen über die Aetiologie des Scharlachs. Verlag der Konigl. Akademie, Berlin, 1905. Also Münch. Med. Wochen., 1905, LII., 1323, 1384, 1574.

Sorensen—Uber sogenannte "return cases" durch Scharlach. Therapeut, Monatsch., 1906, XX., 111.

Stickler, J. W.—Scarlet Fever produced by inoculation, etc. Tr. Med. Soc. N. Jersey, 1897, p. 201.

Sydenham—Omnia Universa. 1685. Londini, W. Kettibly. First English translation by John Pechy, 1717, London, M. Wellington.

Thompson, R. L.—Protozoa-like bodies described in scarlet fever. St. Louis Courier, Med. 1905, XXXII., 351.

Tileston, W., and Locke, E. A.—The blood in scarlet fever. Journ. of Infect. Dis. 1905, II., 375.

Weaver, G. H.—The vitality of bacteria from throats of scarlet fever patients. Journ. Chicago Path. Soc., 1901, V., 259.

SOME CHARACTERISTICS OF THE STREPTOCOCCI FOUND IN SCARLET FEVER.*

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The following work was carried on to determine the frequency of the occurrence of the hemolysing streptococcus in the blood, pus, blister fluid and throats of scarlet fever patients, its relative proportion to other streptococci present and the persistence of its hemolysing power. This study comprises a portion of the preliminary work upon the etiology of scarlet fever which is under investigation at the laboratory. By the term "hemolysing streptococcus" is meant that spherical or spheroidal organism which, by dividing in only one plane, grows in long or short chains, the individuals usually occurring in pairs, and which gives a more or less definite area of hemolysis about each colony when plated out in blood agar poured plates.

In 1903 Schottmüller, and soon after, Rosenow, showed that pneumococcus colonies gave green color in blood agar poured plates, streptococcus pyogenes gave hemolysing colonies and other streptococci (*streptococcus viridans*), green colonies. Reudiger (1906),† working with various throat cultures, found that pneumococci showed green and sometimes hemolysing colonies; streptococcus pyogenes, hemolysing colonies; other streptococci, green colonies. He also found that hemolysing colonies may show green color in subsequent plates, and that slightly hemolysing colonies and those with greenish cast, while very puzzling, should be distinguished from streptococcus pyogenes. Moreover, he found but a small proportion of the green colonies occurring in scarlet and other throat cultures to be pneumococcus. The others seemed to belong to a large group lying between streptococcus pyogenes and the pneumococcus, some being very like the streptococcus viridans of Schottmüller and others related to pneumococci.

Reudiger explains the green coloration in blood plates as due to the formation of lactic acid, which then acts on the sugars in the blood medium. In glucose-agar blood plates streptococcus pyogenes does give green colonies entirely, but none in blood and plain agar because it does not ferment muscle sugar readily and the hemolysis outruns the green coloration. In our laboratory all the hemolysing strains, and also the green ones, when tested and re-tested in glucose agar blood poured plates gave green colonies.

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* This work was carried out under the direction of Drs. W. H. Park and A. W. Williams.

† Journal Infectious Diseases, 1906, Vol. III., p. 755.

The plate method was the chief means of isolating the streptococci in pure culture from the scarlet fever material. In the case of *blood* about 5 or 6 c.c. were drawn from the veins of the arms of living patients, and in autopsy cases from the heart, by means of a sterile syringe. One c.c. was added to a test-tube of melted agar, which had been cooled to 41 degrees C., and the whole poured quickly into a sterile petri dish. The rest of the blood drawn was inoculated into a bottle or flask containing about 75 c.c. of broth, either plain or calcium (containing a few lumps of broken marble). The plate and broth were incubated twenty-four hours at 37 degrees C. If colonies appeared in the blood poured plates they were fished to blood agar slants. The broth was examined microscopically by smears stained with Loeffler's methylene blue, and also plated out by streaking a loopful over the surface of a freshly poured agar plate on which had been placed a loopful of sterile horse blood. The isolated colonies thus obtained were easily fished under the microscope to blood agar slants and tested shortly in blood poured plates, as follows:

From the agar slant a serum broth culture was made and after twenty-four hours incubation at 37 degrees C., dilutions were made by putting 0.25 c.c. in a second broth tube and of this mixture 0.25 c.c. in a third broth tube, and so on up to four or five dilutions. Then 0.1 c.c. from each of the latter dilutions was used to inoculate test tubes, each of which contained 1 c.c. of sterile horse blood. The plates were poured by adding to an inoculated blood tube a tube of melted agar already cooled to 41 degrees C. This gives a better mixture than pouring the blood into the melted agar as is necessary when a syringe is used as above.

Blister Fluid was obtained by applying strong ammonia to a small area of the skin where the rash was brightest, usually on the chest, by means of a disk of blotting paper surrounded by a circle of vaseline and held firmly in place by crossed adhesive strips arranged in a star form, so that the air was practically excluded. After 10-15 minutes the fluid in the blister thus formed was drawn with sterile capillary pipets which penetrated the delicate epidermis easily. Cultures were made in serum broth and, after twenty-four hours incubation, treated as the blood cultures above.

The *pus* was drawn by a sterile syringe from suppurating glands or other foci of infection. Dilutions were made as above, but were plated out at once because the number of organisms present was usually much greater than in the blood.

Throat cultures were taken by means of sterile cotton swabs rubbed thoroughly on both tonsils. The swab of each case was beaten in a tube of plain broth and dilutions made as described before. At first ordinary broth tubes containing varying amounts of fluid were used and these incubated twenty-four hours before the blood poured plates were made. Later, tubes containing exactly 5 c.c. of broth were found more useful and 0.1 c.c. transferred from one to another. The dilutions thus made were plated out at once. It was found, by examining smears of the incubated tubes, that in the higher dilutions the streptococci showed a tendency to outlast other bacteria, so that in about the seventh or ninth dilution one had frequently a pure culture of streptococcus.

The following table gives in condensed form the number of cases examined and strains isolated in pure culture, together with the combined percentage of total number of streptococcus colonies present in the original material from the throats of early scarlet and measles cases.

Streptococci in Scarlet Fever.

Source.	Number of cases tested.	No growth.	Strept. isolated.	Blood Pour Plates.	
				Strept. Pyogenes (Hemolysing).	Other Strept.
Autopsy blood (from heart) ..	18	8	10	+	—
Living blood (from arm)	16	15	1	—	+
Total............	34		11		
Autopsy pus.................	10	3	7	+	—
Living pus....................	9	0	9	+	—
Total	19		16		
Blister Fluid.................	26	23	3	—	+
Throats (scarlet).............	35 (5 rejected.	0	30	+ 27.3%*	+ 72.7%*
Controls: Measles Throats..	24	..	24	14.4%	85.6%
Diphtheria Throats..........	4	..	4	Fraction of 1%	+

* The calculation of a *percentage* of hemolysing colonies was begun only in the last half of the scarlet throats. but was carried out in all measles throats tested and varied considerably as follows:

Percentage of Hemolysing Colonies in Individual Cases.

Scarlet Throats.		Measles Throats.	
15 Cases.	Percentage.	24 Cases.	Percentage.
Scarlet fever 79	100.0	Measles 13	10.0
" 80	0.0	" 14	50.0
" 81	0.2	" 15	4.0
" 82	0.7	" 16	2.0
" 83	10.0	" 18	33⅓
" 84	7.5	" 19	2.0
" 85	33⅓	" 20	0.0
" 86	0.0	" 21	25.0
" 87	0.0	" 22	0.0
" 88	80.0	" 23	14.0
" 89	17.0	" 24	1.0
" 90	70.0	" 25	0.0
" 91	91.0	" 26	0.0
" 96	40.0	" 27	3.+
" 97	50.0	" 28	87.0
		" 29	14.0
		" 30	2.0
		" 31	16.0
		" 32	20.0
		" 33	2.2
		" 34	60.0
		" 35	0.6
		" 36	0.0
		" 37	0.3

When the throat cultures of scarlet fever, measles and diphtheria were isolated by means of blood poured plates, the streptococci found showed considerable variation in reaction and the following terms were used in an attempt to classify the colonies:

1. Hemolysing, represented in charts by "A."
2. Small Hemolysing, represented in charts by "a."
3. Very Small Hemolysing, represented in charts by "a_1."
4. Slightly Hemolysing (large and small), represented in charts by "b."
5. Doubtful, represented in charts by "c."
6. Green (light and dark), represented in charts by "d."

Colonies were fished and replated during successive generations and it was found they varied up and down the scale:

Hemolysing colonies varied in area of hemolysis.

Green colonies varied in shade of green.

Small hemolysing colonies became *large* or *slightly hemolysing*, or *green;* this green in subsequent generations again became hemolysing.

Slightly hemolysing colonies remained the *same*, became *doubtful* or showed *green* in later plates.

Doubtful colonies became *slightly hemolysing*, remained *doubtful* or showed *green*.

Thirty streptococcus strains isolated from blood, pus, and blister fluid of scarlet fever cases were studied.

Autopsy Cases.		Living Cases.		
Case No.		Case No.		
13	Blood. Pus.	211	Blister.	
50	Blood.	226	Blister.	
53	Blood. Pus.	Sc. 22	Blister.	
96	Blood. Pus.	51	Blood. Throat	
204	Pus.	Sc. 93	Pus.	Cervical glands.
206	Blood.	Sc. 95	Pus.	
212	Pus.	Sc. 98	Pus.	
Sc. 23	Blood.	Sc. 99	Pus.	
Sc. 24	Blood. Pus.	Measles and scarlet fever 102	Pus.	
Sc. 30	Blood.	Sc. 103	Pus.	
Sc. 48	Blood.	Sc. 104	Pus.	
Sc. 92	Pus.	Sc. 105	Pus.	
		Sc. 106	Pus.	

Controls: No. C. 100 Bellevue Laboratory Culture.
No. C. 101 Cellulitis after erysipelas.

With one exception every *blood* and *pus* case in pure culture when tested in plain agar blood poured plates gave good hemolysing colonies. The *exception was the only strain isolated from living blood, and it gave green colonies.* The throat culture from this case also showed green colonies only. The patient from whom these were derived had a good rash, ran a typical course of scarlet fever and recovered *without complications.*

The three *blister fluids* which yielded streptococci showed both hemolysing and green colonies.

Re-test of Hemolysing Power of Above Strains.

The strains enumerated above (with the exception of Sc. 102, 103, 104, 105 and 106, which have only recently been isolated), and the two controls, making 27 strains in all, were kept in the ice-box on blood agar slants (being transferred at about four-week intervals), for three months to a year. They were then retested in successive blood poured plates and *showed marked variation as to hemolysis*, though all, except 51 Blood and Throat (which were green when isolated and remained so), had been fished from colonies which showed good hemolysis in the original. The re-test was carried on as follows:

From a 24-hour blood agar slant of each strain a tiny portion of the growth was removed with a straight platinum needle and put in a 5 c.c. broth tube. This was shaken thoroughly and the two loopfuls of the broth transferred at once to a tube containing 1 c.c. of sterile horse blood. Melted agar was added and the plated poured as before. These plates were incubated eighteen to twenty-four hours and the colonies showing least amount of hemolysis were fished on blood agar slants which were plated out in turn. A careful record was kept as to the size and reaction of the colonies of each strain, only the most variable fishings being carried on from one plating to another. Each broth tube, from which an agar plate was made, was incubated and a smear from it examined, so no contamination could creep in unawares.

From the *hemolysing* colonies arose:

1. Hemolysing colonies of similar and smaller size.
2. Slightly hemolysing (large and small).
3. Doubtful colonies.
4. Green (light and dark).

In subsequent platings, as in the case of fishings from the throat cultures, there was a marked variation up and down the scale (see charts).

Hemolysing colonies varied in hemolysis, became slightly hemolysing, doubtful, green or showed no green nor hemolysis.

Slightly hemolysing colonies became hemolysing, remained slightly hemolysing or were doubtful, green or showed no green or hemolysis.

Green colonies remained green for the most part, but a few (see charts), returned to hemolysing, slightly hemolysing or were doubtful.

In two strains, 53 *Blood* and *Sc.* 48 *Blood*, at the fourth and third platings, respectively, there occurred among other colonies some which showed *neither hemolysis nor green color*. These were carefully fished and found in smears of broth transfers to be pure streptococcus. When these fishings were replated and refished one or more times they gave rise to *hemolysing, slightly hemolysing, green* and *doubtful colonies,* sometimes all in the same plate. Only a few remained the same, that is, showed no green nor hemolysis.

In the accompanying charts of *53 Blood and 53 Pus and *Sc. 48 Blood the variation is expressed as follows:

A+ = Large hemolysing—4-5 m.m. in diameter.
A = Medium hemolysing—3 m.m.
A_1 = Less hemolysing—2-2-½ m.m.
a = Small hemolysing—1-½ m.m.
a_1 = Very small hemolysing—1 m.m.
b+ = Slightly hemolysing (large).
b = Slightly hemolysing.
c = Doubtful.
d_1 = Light green.
d = Green.
d+ = Dark green.
E = No green, no hemolysis.

The figures above the letter (in the charts) indicate the succession of platings. These were usually at intervals of two or three days.

Eight fishings from colonies (of 53 Blood), which showed no green and no hemolysis were tested simultaneously in blood pour plates of plain agar, peptone water agar, and sugar-free agar (made from broth with colon bacillus added and re-sterilized), with the result that in the plain and peptone agar the hemolysis returned for the most part, while in the sugar-free agar the colonies remained the same or showed but slight trace of hemolysis.

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* The charts of *Sc.* 48 *Blood* and 53 *Blood* were so complicated that for the sake of clearness only a part of each chart is reproduced; the parts are typical, however, of the many fishings which were carried on from these same strains. The 53 *Pus* chart is a simple one and is given entire. It is typical also of the other cases tested.

Blood Pour Plates.

Fishings of 53 Blood.	Plain Agar.	Peptone Agar Watery 1%.	Sugar-free Agar.
(1) E..................	E + b 60%	A (+ b)	E (+ b) 80%
(2) E..................	A	A + 2	E (+ b) 90%
3) E..................	A	A (+ b) 96%	E (+ b) 99%
(4) E..................	E (+ c) 95%	A (+ b) 90%	E
(5) E..................	A (+ b) few	A (+ b) 90%	E (+ b) few
6) E..................	A—(+ b) few	A—(+ a)	E
7) E..................	A (+ b) 80%	A (+ b) few	E (+ b) few
8) E..................	a	a	E
* Control.			
204 Pus d..............	d—	d—	d—

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* The control in this test was a fishing (from 204 pus) which had remained green through five platings, and showed green colonies in all three media.

These "E" colonies (showing no green or hemolysis) were tested also upon:

Glucose agar which gave *green* colonies.

Glycerine agar which gave hemolysing and slightly hemolysing colonies.

Beerwort agar which gave deep green colonies.

Two strains, 13 Blood and Pus, were kept on glucose agar blood slants for a number of transfers at several day intervals and when changed suddenly to plain agar blood pour plates showed no loss of hemolysing power.

The two control cultures, one C100 from the laboratory of Bellevue Hospital and the other C101 isolated recently from a case of cellulitis following erysipelas, showed but little variation, for only two slightly hemolysing colonies occurred in the six platings of C100 and none in C101.

A re-test was made also of 20 fishings from *scarlet throats* as follows:

Hemolysing.	Slightly Hemolysing.	Green.
Scarlet fever, 24.		
Scarlet fever, 79.		
Scarlet fever, 81	Scarlet fever, 81.	
Scarlet fever, 82		Scarlet fever, 82.
Scarlet fever, 83.		
Scarlet fever, 84.		
Scarlet fever, 85 (small).		
Scarlet fever, 88	Scarlet fever, 88 (large)	Scarlet fever, 87. Scarlet fever, 88. Scarlet fever, 89.
Scarlet fever, 90.		
Scarlet fever, 91 (small).		
Scarlet fever, 96.		
Scarlet fever, 97.		
Scarlet fever, 211.		
Scarlet fever, 212.		

Control Sc. 40 = from erysipelas case which developed rash and was suspected of being scarlet fever, but later proved negative.

These strains had been kept in the ice-box on blood agar slants for 12-18 months, and comprised not only hemolysing, but hemolysing and green colonies when first isolated.

The reactions of the originally *hemolysing colonies* followed practically those showed of the blood and pus hemolysing colonies when they were re-tested.

The colonies which had been *green* when isolated, remained so in every re-plating except once in the case Sc. 87, where doubtful colonies occurred in the third plate, but when these were fished and plated again they showed green and remained so for subsequent platings.

In the replating of the two *slightly hemolysing strains, Sc.* 81 *and Sc.* 88, greater variation occurred as may be seen by the accompanying charts which show part of the fishings carried on in each case.

88 *slightly hemolysing (large)*, gave: hemolysing, slightly hemolysing and a few doubtful colonies which, however, became hemolysing again. On the other hand 81 *slightly hemolysing* gave but a low per-

centage of hemolysing colonies and these were small in size. The majority of colonies were slightly hemolysing with a few doubtful and a number of green ones. These last, the green, became small and tiny hemolysing colonies four times, but for the most part gave rise to slightly hemolysing and still other green colonies (see chart).

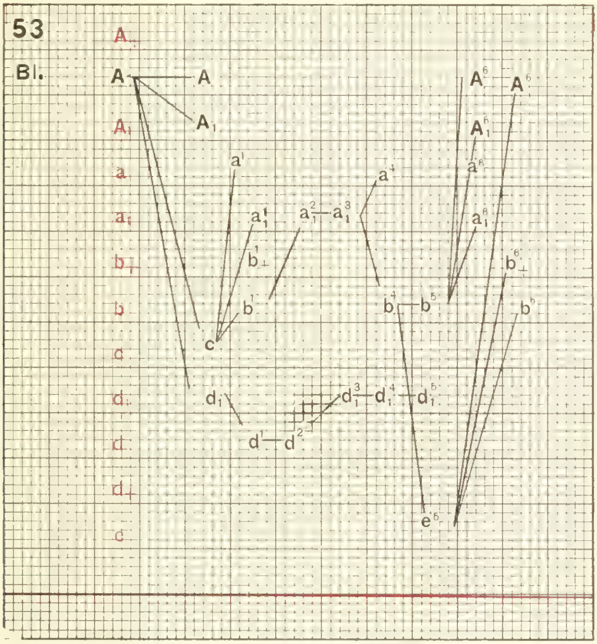
The *control* from the erysipelas throat which was hemolysing when isolated, gave in the re-test, *large* hemolysing colonies.

Although the power of hemolysing streptococci to cause hemolysis seems to be a slightly variable quality and is certainly dependent upon conditions we do not understand, yet there is a distinct tendency of this power to continue, even though the strains be kept on artificial media, for in the re-test the majority of colonies were hemolysing, the variation from the original strain being usually about 5 per cent., or less, in the first re-plating. In these and subsequent plates only the most variable colonies were fished and even then the percentage of hemolysing colonies was usually greater than 25 per cent., sometimes running between 75 and 100 per cent. All strains were plated out at least six successive times and some, eight or nine times. Any variation occurring cannot be due entirely to long cultivation on artificial media, partly because some recent green colonies derived from old hemolysing cultures kept on agar for seven to twelve months have, as is shown in the chart Sc. 48 Blood, regained their hemolysis once more. To be sure, when some of these apparently accidental green colonies, which formed but a very small part of all the colonies present, were selected strains were brought out which had lost their hemolysis completely. This occurred in 33 1/3 per cent. of the 27 blood and pus cultures retested, and these green strains failed to regain any appearance of hemolysis, though they were plated out six successive times (see chart of 53 Blood and Pus). It is doubtful if these can longer be classed with *streptococcus pyogenes.* On the other hand, as cited above in the chart of Sc. 48 Blood, some green colonies show a quick change to hemolysis again.

The occurrence of hemolysing streptococci (strep. pyogenes), as seen in the table, was almost constant in the material taken from scarlet, measles and diphtheria throats, while the streptococcus viridans was always present. In scarlet cases the hemolysing streptococcus alone occurred in the pus from cervical glands and other suppurating foci in all of the nine living cases, and was recovered in seven of the ten autopsies. In the living blood and blister fluid streptococci would seem to be rather infrequent since they were found in but three out of twenty-six blisters and once in sixteen samples of living blood. It is interesting to note that the three children from whom streptococci, both hemolysing and viridans were isolated by blistering and also the man whose blood contained the streptococcus viridians, all recovered. Streptococci were not found in all cases of autopsy blood examined, being present in but ten of the eighteen tested and were of the hemolysing type.

53 Blood.

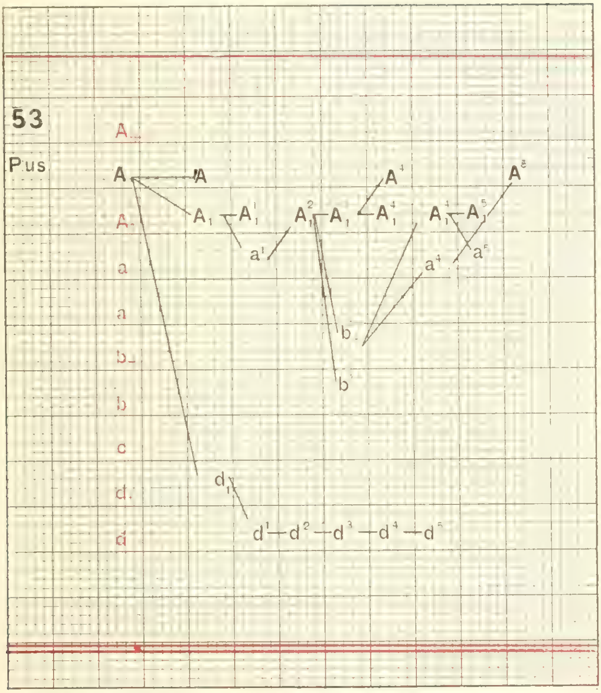
Typical Portion of chart for 53 Blood shortly after death, showing variation in hemolysing power of colonies in successive cultures from a markedly hemolysing streptococcus derived from heart's blood of a scarlet fever case after death.



A+ = large hemolysing.
A = medium hemolysing.
A_1 = less hemolysing.
a = small hemolysing.
a_1 = very small hemolysing.
b+ = slightly hemolysing (large).
b_1 = slightly hemolysing (small).
c = doubtful.
d_1 = green (light).
d = green.
d+ = dark green.
E = no green, no hemolysis.

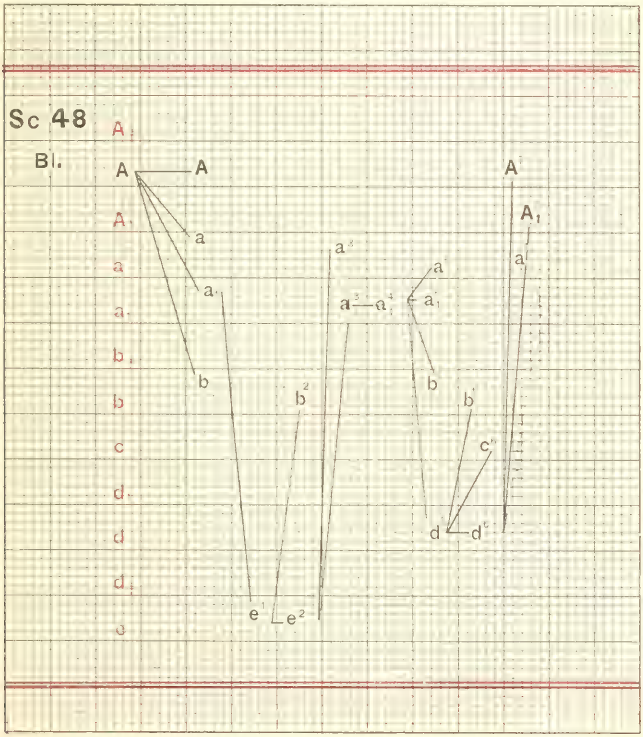
53 Pus.

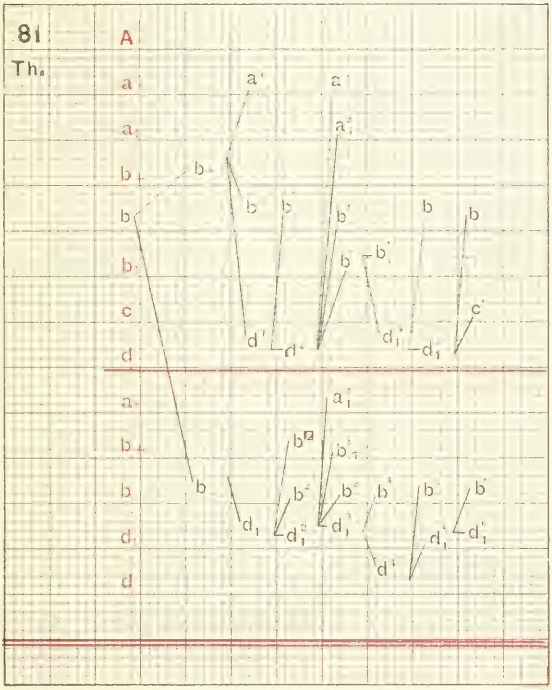
Whole chart, same case as 53 Blood.



Sc. 48 Blood.

Typical portion of chart showing variation in hemolysing power of colonies in successive cultures from a markedly hemolysing streptococcus colony derived from heart's blood of scarlet fever case 2½ hours after death.

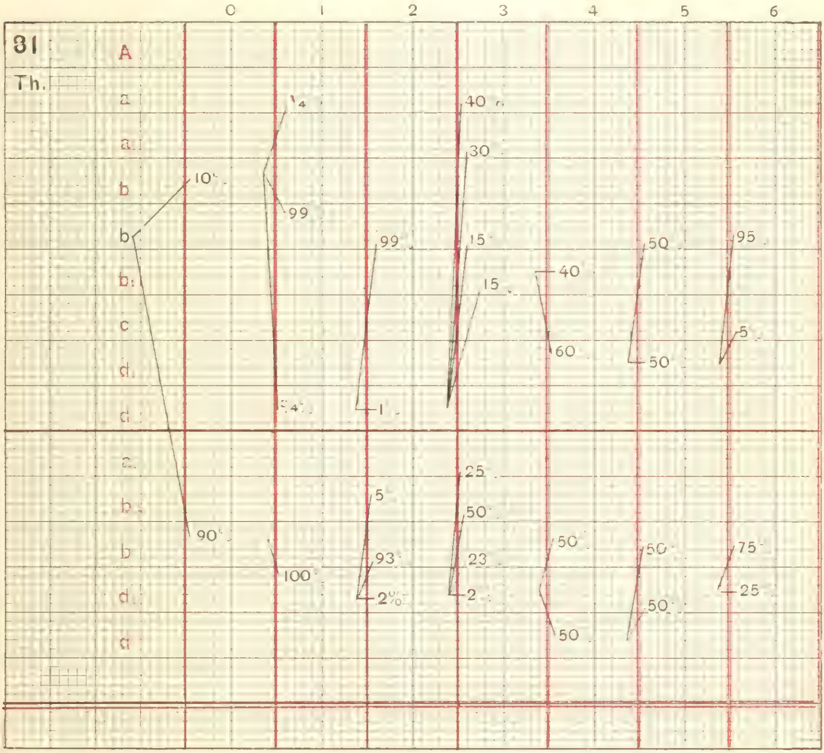




81 Throat. Typical portion.

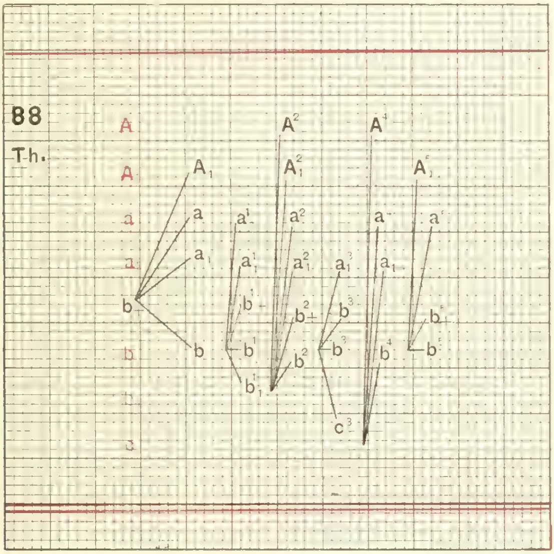
81 Throat.

Duplicate chart, showing *percentage of variation* in hemolysing power of colonies in successive cultures from a *slightly* hemolysing streptococcus colony from throat of scarlet fever cases.



88 Throat.

Typical portion of chart showing variation in hemolyzing power of colonies in successive cultures from a slightly hemolysing streptococcus colony from throat of scarlet fever case.



ROUTINE DIAGNOSIS OF RABIES IN THE LABORATORY OF THE NEW YORK CITY HEALTH DEPARTMENT DURING THE YEARS 1906 AND 1907.

By Anna W. Williams, M. D.

In the Research Laboratory, for the past two years, or since we have used the smear method in the routine diagnosis of rabies, there have been over 700 cases in all examined, including suspected rabies and controls.

These are divided into two groups, the first comprising the cases sent in for diagnosis, and the second the experimental cases.

When we began the work, in order thoroughly to test the smear method in diagnosis we used comparative tests on a long series of cases, including many diseases other than rabies. The comparative tests were animal inoculations and a study of sections. The results obtained from a study of 141 cases were published in January and May, 1906,* and our conclusions were that the Negri bodies as shown by smears, as well as by sections, are specific for hydrophobia, and that the smear method for examining the Negri bodies is more rapid and efficient than any other method published. Since then, in our routine work we have considered the presence of the Negri bodies in smears as diagnostic of rabies and have made no further control tests, except in those cases which we have used in our experimental work. Through this experimental work, however, we have added 119 cases to the list of those which had the comparative tests and our former conclusions have been more firmly established.

The following table gives the number of suspected animals sent for diagnosis each month during the years 1906 and 1907. Since, generally, only those animals that have bitten people are sent in for diagnosis, many cases of dumb rabies and of rabies with atypical symptoms are not diagnosed. Therefore, we can judge only roughly from these statistics as to the prevalence and increase of rabies in New York and vicinity.

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* See in Collected Studies from the Research Laboratory, Vol. II., 1906.

TABLE I.

Animals Sent to Research Laboratory for Diagnosis.

Month.	1906.		1907.	
	Positive.	Negative.	Positive.	Negative.
January	7	1	18	3
February	5	1	12	6
March	9	1	12	2
April	5	1	9	5
May	12	1	13	3
June	10	4	11	7
July	3	7	10	5
August	9	5	19	6
September	7	2	21	8
October	9	8	14	5
November	11	1	24	4
December	14	5	18	5
Total	101	37	181	59

According to this table there has been a decided increase in the number of cases during 1907, but in interpreting these figures we must bear in mind that people are realizing more and more, especially since several prominent citizens have died from typical rabies, that such a disease really exists, and therefore, they are more particular about sending in material from suspicious cases. In the following table the animals received for diagnosis during 1907 are arranged according to the species and to the number of cases within and without the city.

TABLE II.

Kinds of Animals Received for Diagnosis During 1907.

Kind.	Locality Whence Received.			Diagnosis.	
	In City.	Out of City.	Total.	Positive.	Negative.
Dogs	187	31	218	168	50
Cats	7	2	9	4	5
Human beings	5	1	6	6	..
Horses	4	..	4	2	2
Cows	..	2	2	..	2
Pigs	..	1	1	1	..
Total	203	37	240	181	59

Before analyzing the 573 cases examined since May, 1906, it may be well to state that by "Negri bodies" we do not mean the indefinite cell inclusions such as those found by Van Gieson in human meningitis, or by a few others in cats' brains, or by ourselves in dog and cat distemper. The more such indefinite bodies are found in other diseases, the greater is the evidence in favor of the specificity of the true Negri bodies.

Of the 537 cases, 335 are street cases and 238 experimental. Of the 335 street cases, 241 show typical Negri bodies and 94 show no definite forms. All of the latter, classed as doubtful cases, and 36 of the former were inoculated into animals. The 36 all gave positive results.

Of the 94 classed under doubtful cases, 12 brains were too decomposed to show anything; 4 of these were positive on animal inoculations; 10 were soft and showed suspicious bodies; 3 of these were positive; 7 were in good condition and showed suspicious bodies, 4 of these were positive, the 65 remaining ones showed absolutely no suspicious bodies and all were negative to animal inoculations.

We see, then, that in all of our work controlled by careful animal inoculations we have never yet failed to have typical rabies develop in animals inoculated with material showing definitely-structured Negri bodies.

Negative results after inoculation with such material must be interpreted by us at present as due to some error in technic, such as regurgitation or hemorrhage at the time of inoculation, emulsion improperly made, not enough of the virulent material taken because of localization of the organisms, etc. Possibly individual resistance of the animal inoculated might play a part. We have used principally guinea pigs, and some of them have shown enough irregularity in regard to the time in which they have come down with the disease to suggest a varied individual susceptibility, if other factors can be ruled out.* So far, however, as I have stated, we have gotten no negative results with material showing Negri bodies.

No one has yet reported the finding of the characteristic structured bodies in any disease other than rabies. We give in the bibliography a list of works relating to the diagnosis of rabies which have appeared since May, 1906, as well as a few articles which were not included in our previous list (see J. Inf. Dis., 1906, Vol. III., 452). Lentz and Bohne have corroborated our methods of demonstrating the bodies. And all authors still agree that the typical Negri body is absolutely diagnostic of rabies.

On the other hand, some material in which we have failed to demonstrate typically-structured bodies has produced rabies. All of this material, however, since we improved our technic, has shown suspicious small forms similar to those found in rabbit fixed virus. But any decomposing brains may also show in smears, bodies very similar to these tiny forms, therefore, it is difficult to rule out rabies in such cases. Such cases have become less frequent with the improved technic, and if, as we believe, the Negri bodies are living organisms, there is strong reason to hope that in time we may clearly demonstrate in smears the presence of these tiny specific forms.

Until such a time, however, animal inoculations must be used in these cases. Of course, the animal test will probably always have to be used with brains that are too decomposed for the recognition of any formed elements except bacteria, unless a reliable chemical test can be discovered.

So far with us, *fresh* brains showing no Negri bodies and no suspicious forms have not produced rabies, but a few observers have claimed that such material has produced the disease. Therefore, until we can reach a unanimous conclusion in regard to this point, we must in all such cases use animal inoculations. We may, however, be reasonably certain that a case showing such negative material was not a case of rabies.

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* The inoculations have all been made by the same operator, Mr. T. Deaken, and this may be the chief reason why our results with guinea pigs have been so uniform.

It is possible that some of the animals from which this doubtful material was derived, killed early in the disease, before there had been time for general development of the virus in the brain, may have had virulent sputum. The virulence of the sputum is said to be due to the passage of the virus through the salivary glands. We are now carrying on a series of experiments to test the comparative virulence of these glands and the brain in true and doubtful cases. Our results are yet too few to be tabulated. We have, however, already had an interesting case in which this method was apparently helpful. A Russian wolfhound with typical symptoms of dumb rabies was sent in for diagnosis. The brain showed many small fixed-virus-like forms, which were considered suspicious. Guinea pigs were inoculated from brains and glands. Treatment was begun in a small child whose scratched hand had come in contact with the saliva. After twelve days, as no symptoms were observed in the guinea pigs, the treatment was discontinued. The pigs from the brain came down six and one-half weeks after the inoculation, while those from the glands are still alive ten weeks later. It was decided not to give more treatment to the child for this reason.

The question as to whether the *sputum* may be virulent and the salivary glands not, is an interesting one. It has been stated that the secretions from the mucous membranes of the mouth and naso-pharynx may also be virulent. If this were so, it would of course throw out negative results from gland inoculations. This question is now being investigated.

The localization of the Negri bodies is another important point in making diagnoses. We have found well-developed bodies distinctly localized in different parts of the brain in several instances. In one horse there were small, widely-scattered areas of well-structured forms throughout the cerebellum, while tiny, indefinite forms were scattered through the rest of the brain examined. In two human brains well developed forms were found in the corpus striatum and not in the rest of the brain. In several dogs the localization has also been marked.

The full technic of our present method of examination is as follows:

(1) The whole brain, and when wished, the Gasserian ganglion, spinal cord and its ganglia are removed under sterile precautions.

(2) The salivary glands are removed under sterile precautions.

(3) Small sections are cut from the various parts desired, especially from ammon's horn, cerebellum, and motor area of cortex. The sections are fixed in Zenker's fluid and embedded in paraffin, according to the method given in previous article.*

(4) Smears are made by pressing between a glass slide and a cover glass a small, thin section of the gray matter from (a) the cerebral cortex, (b) ammon's horn, or (c) the cerebellum; the material is spread along the slide by moving the cover glass down with the finger.

When partly or completely air-dried, the smears are fixed for about ten seconds in neutralized† methyl alcohol to which one-tenth per cent. of picric acid has been added. The excess of the fixative is removed by blotting with fine filter paper.

The fixed smears are stained with the following solution:

Saturated alcoholic solution of fuchsin	0.3 c.c.
Saturated alcoholic solution of methylene blue . .	2.0 c.c.
Distilled water	30.0 c.c.

NOTE—This solution changes rather quickly at room temperature, but kept in the icebox it gives good results for an indefinite time.

The stain is poured on the smear and held over the flame until it steams. The smear is then washed in tap water and blotted with fine filter paper.

With this stain the Negri bodies appear a magenta, the nerve cells blue, and the red blood cells yellow or salmon color.

(5) If nothing is found in the smears from the parts mentioned, smears are made from various other parts of the brain. If still nothing is found, an emulsion is made of good-sized pieces from the different

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* Journ. of Inf. Dis., May, 1906.

† The wood alcohol is neutralized by adding sodium carbonate (Na_2CO_3) about .25 gms. to 500 c.c. of the alcohol.

‡ This mixture is practically the same as that recommended by Van Gieson for staining the Negri bodies.

parts of the brain and intracerebral inoculations are made into three guinea pigs, about one-fourth c.c. of the emulsion is inoculated. An emulsion is also made of the different parts of the brain in glycerine for later inoculations, if for any reason the first should fail. Sterilized, neutral glycerine is used. These emulsions remain active in the ice-box for over three months.

If one has a freezing ice-box, the whole brain may be frozen, in which condition it will remain in a perfect state of preservation and fully virulent for over three months.

(6) Contaminated, doubtful material is made into a weaker fresh emulsion in order to lessen the number of bacteria. The glycerinated emulsion made from part of the same material is inoculated after two weeks, unless positive results have been gotten from the weak solution.

(7) One of the three guinea pigs is killed on the eighth day after inoculation, when, if the material inoculated was rabic the animal may show definite Negri bodies in the brain. Of course negative results are not definite. The other animals, if they do not develop rabies, are kept under observation for from four to six months.

Bibliography.

Babes, V.—Ueber die Negrischen Körperchen und die Parasiten der Tollwut. Romania Medicale, 1906.

Babes, V.—Les Corpuscles de Negri et le Parasites de la Rage. Presse Med., 1906, p. 669.

Babes, V.—Untersuchung über die Negrischen Körperchen und ihre Beziehung zu den Virus der Wutkrankheit (Zeit. Hyg., LVI., 1907, 435).

Baschieri.—Sulla Diagnose rapida della rabbie. Soc. Med. Charm di Biologie, 1906.

Bohne, A.—Die Negrischen Körperchen und ihre Bedeutung für die Diagnos der Tollwut. Ztschr. f. Infect. par. Krank. u. Hyg. der Haustiere, 1907, II., 229.

Bongiovanni, A.—Die Negrischen Körperchen und die durch fixes Virus verursachte Wutinfection mit langsamen Verlauf. Centralbl. f. Bakt. Orig., 1908, 41.

Calkin, G. N.—The protozoan life cycle. Biological Bulletin, 1906, XI.

Davis, D. J.—Negri bodies in Hydrophobia. Journ. Am. Med. Association, 1906, 47, p. 87.

Fermi, C.—Die Cerebrospinal-flüssigkeit wutkranker Tiere ist nicht virulent. Fermi, C. Ueber die Virulence des Speichels u. d. Speicheldrüsen wutkranker Tiere (Centralbl. f. Bakt. I., orig. XLIV., 1907, 25 and 26).

Fursenko, B.—Ueber die Negrischen Körperchen im Virus fixe. Centralbl. Bact. Orig. Bd. XLIII., 360.

Heller, Otto—Die Schutzimpfung gegen Lyssa. Jena, 1906.

Lentz, Otto—Ein Beitrag zur Färbung der Negri 'schen Körperchen. C. B. I. Orig., 1907, XLIV., 374.

Henke, N. & Zeller—Centralbl. f. Path. Anat., 1905, 2.

Manouelian, T.—Note sur l'existence des produits de degenerescence cellulaires rappelant les corps de Negri; Des Comptes Rendus de L'Academie des Sciences. Feb. 24, 1908.

Mazzei, Tullio—Sulla resistenza del virus rabbico alla putrofazione (Riforme Med., 1906, No. 38, p. 1050).

Negri, A.—Sulla Morfologia e sul Ciclo Evolutivo del Parassita della Rabbia. Roma, Tipografia della R. Academia dei Lince, 1907.

Pate, C. W.—Rabies in a goat (Journ. of Trop. Vet. Sci., I., 1906, 419).

Pfeiler, Willy—Beitrag zur Darstellung der Negrischen Körperchen Ztsch. f. Infect. parasit. Krank. u. Hyg. d. Haustiere, 1907, II., p. 388.

Stefanescu, Elise—La presence des corpuscules de Negri dans les glandes salivaires des chien enrages. Compt. rend. Soc. biol., 1907, LXII., No. 17.

Tizzoni, G. & Bongiovanni, A.—Ueber den Mechanismus des Zerlegung des Wut virus in vitro durch das Radium (C. B. I. Orig., 1907, XLIV., 353).

Strazzi—Das Negrischen Körperchen u. die Schnell-diagnose der Wut. La clinica Vet., 1904, 42.

Williams & Lowden—The etiology and diagnosis of hydrophobia. Journ. Inf. Dis., 1906, 452.

Zaccaria—Sullo prestice e distributione dei corpi dei negri. Ann. d Igiene. Sperimentale, 1905, 15.

Zeller—Zentralblatt f. pathol. Anatomie und allg. Pathol., 1905, Nr. 2.

THE QUANTITATIVE CHANGES, DURING IMMUNIZATION, IN THE BLOOD OF HORSES, AND THE RELATION OF THE SERUMGLOBULIN TO DIPHTHERIA AND TETANUS ANTITOXIN CONTENT.

Drs. Edwin J. Banzhaf and Robert B. Gibson.

Gravimetric determinations were recorded for the total and several individual proteins of sodium oxalate plasma fractioned with ammonium sulphate and sodium chloride. When precipitated the plasma-salt solution had a resulting volume of ten times the amount of the plasma employed. Coagulations were on aliquot portions of the filtrates, and the protein constituents (except serum albumin and in part the saturated NaCl soluble serum globulin), were calculated by difference. The eleven horses were subjected to an immunization, under Dr. Park's direction, against diphtheria and tetanus toxins simultaneously, each animal subsequently being continued on the toxin to which it responded best. Test bleedings, of about 500 c.c. only, were made until maximum antitoxic potency (with almost coincident greatest variation in content of the proteins), had been attained; afterwards routine bleedings were undertaken. The two refractory, one medium, and the eight horses producing a highly potent antitoxin all showed a maximum increase of from 40-114 per cent. for the serum globulin (for the refractory horses, 59.3 and 89.8 respectively). In one refractory and one highly antitoxic horse, the serum globulin maximum preceded the highest concentration in antitoxin; with three other horses, both maxima were attained when examined on the second bleeding. In seven of the horses, the greatest content in serum globulin was coincident with the maximum antitoxic potency. The greatest content in serum globulin was observed in the most potent plasma obtained in the series. The serumglobulin subsequently was maintained at a high concentration, roughly paralleling the antitoxic content in the plasma of the individual animals. The serumalbumin was diminished a half to a third along with the serumglobulin increase; though subsequent to the antitoxic and serumglobulin maxima, figures as low as a fifth the original albumin content were noted. The saturated NaCl soluble serumglobulin was relatively increased from a normal 60-80 per cent. of the total serumglobulin to over 90 per cent.; in quantity, there is a rise of over 100 per cent., an increase of 163 per cent. being observed in one of the refractory horses. At dilutions of the plasma in the precipitated mixtures of 1:1.5, 1:5 and 1.10, the "euglobulin fraction" amounted to 60-70, 20-24 and 10-15 per cents. respectively of the total serumglobulin in both the normal and antitoxic plasma. In the 850 unit plasma, an increase in the "euglobulin" was noted, but the high content of proteins had probably influenced the precipitation limits. It would seem, then, that the "euglobulin" is not increased relatively to the total serumglobulin during the immunization as has at times been maintained. From the results we have obtained, it may be concluded that in forced immunization, the same characteristic quantitative changes can occur in the blood proteins, both of refractory horses and of those yielding a highly potent antitoxin.

THE FRACTIONAL PRECIPITATION OF ANTITOXIC SERUM.

By Drs. Edwin J. Banzhaf and Robert Banks Gibson.

Comparatively little attention has been paid to the fractional precipitation of antitoxin. Brodie,[1] in 1897, separated antitoxin horse serum into four fractions by the progressive addition of ammonium sulphate of half saturation; all four contained, however, relatively equal amounts of antitoxin. Atkinson,[2] in this laboratory, saturated with sodium chloride a solution of the moist serum globulin precipitate obtained with magnesium sulphate, and by then employing heat differentiated the globulin into several fractions containing antitoxin. The protective properties corresponded roughly to the quantities of serum globulin in the precipitates. In some unpublished experiments he found that alterations of the amounts of coagulated proteid in the several fractions resulted if more magnesium sulphate was added before heating; there were proportionate changes in the distribution of the antitoxin. Owing to the destruction of a portion of the antitoxin at the higher temperature and possible injury by exposing it to heat of less degree, this fractionation must be considered as incomplete and does not exclude a purification of the antitoxin by salt fractionation. The work of E. P. Pick on the ammonium sulphate fractioning of the antibodies has been referred to in the preceding communication. Our own experiments have resulted somewhat differently from either those reported by Atkinson or by Pick, and have developed some new and suggestive facts.

On the basis of the solubility of the antitoxic proteids in saturated sodium chloride solution, one of us (Gibson), recently devised a method for the partial purification and concentration of antitoxin.[1] This consisted in precipitating the diluted plasma with an equal volume of saturated ammonium sulphate and separating the antitoxic proteids by extracting the precipitate with saturated sodium chloride solution. We now have employed the method of salt fractionation to study further the concentration of antitoxin.

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[1] Brodie: *Journ. of Path. and Bact.*, iv, p. 460, 1897.

[2] Atkinson: *Journ. of Exper. Med.*, v, p. 67, 1901.

There exists at the present time considerable confusion in comprehending the methods and basic principles of ammonium sulphate fractional precipitation of proteids. The nomenclature which we have employed and which designates the number of c.c. of saturated ammonium sulphate solution in 10 c.c. of the *precipitated* mixture has been used used by some authorities; it avoids the confusion developed by the use of such terms as "per cent $(NH_4)_2SO_4$ solution" and "per cent saturation $(NH_4)_2SO_4$" "per cent of saturated $(NH_4)_2SO_4$ solution" "per cent saturation $(NH_4)_2SO_4$ solution" and it seems the simplest and best *practical* expression of degrees of saturation yet suggested. We advise that this method be employed in future papers on fractional precipitation.

Mann, in his version of Cohnheim's "*Chemie der Eiweisskörper,*" states (p. 292): "As the solubility of ammonium sulphate is 76.8° (per cent?) at room temperature, it is easy to calculate what percentage of ammonium sulphate is required for bringing about incipient and complete precipitation of any one albumin, as soon as we know what amounts of saturated ammonium sulphate have to be added for any given quantity of fluid." The simplicity of the above method of calculating vanishes when attention is drawn to the fact that while 100 parts of water dissolve 76.8 gms. of dry ammonium sulphate, the volume resulting is increased to 141 c.c. so that 100 c.c. of the saturated solution actually contain approximately 54 gms. of the salt, and the degree of saturation as indicated by the *content* of dry ammonium sulphate must be calculated with reference to the latter figure. An example will make clearer the above statement: To obtain a concentration of "half saturation" ammonium sulphate, equal volumes of the proteid solution and of saturated ammonium sulphate solution are mixed; according to the apparent meaning of Mann's obscure statement, 100 c.c. of the resulting "half saturated" solution would contain 38.4 gms. of the dry salt; it actually does contain 27 gms. of ammonium sulphate.[2]

E. P. Pick has fallen into the same error in his paper on the fractionation of the anti-substances in the globulins of serum. He speaks (p. 356) of the limits of the various serum fractions as follows: "dass das von Reye aus normalen Pferdeserum abgeschiedene Fibrinoglobulin entsprechend einer Sättigung von 21.5 Proz. Ammonsulfat........ein bestimmter Teil (the euglobulin) des nun übrig bleibenden Globulins keine antitoxische Wirkung hatte und dass sich dieser aus dem Serum noch bequem abscheiden liess, wenn die Flüssigkeit einer Gehalt von 25.6 Proz. an Ammonsulfat enthielt. Es verblieb nunmehr ein Eiweissköper in Lösung (the pseudoglobulin) der durch weiteres Eintragen der gestättigten Ammon-sulfatlösung bis zu einem Gehalte von 38 Proz. von dem Serumalbumin gut zu trennen ist und den Heilkörper in quantitativer

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[1] The literature on the purification and chemical characters of anti-bodies has been briefly reviewed in a paper on "The Practical Concentration of Diphtheria Antitoxin for Therapeutic Use," this *Journal*, i, p. 161, 1906, and more recently by Ledingham: *Journ. of Hyg.*, vii, p. 65, 1907.

[2] Because of the change in the volume of the solvent on adding the salt, it is similarly not possible to add 38.4 gms. of ammonium sulphate to 100 c.c of water and have a solution at "half saturation." In this case the volume would be increased to 120.7 c.c. and 100 c.c. would contain 31.7 gms. of the salt.

Ausbeute enthält." The precipitation limits are distinctly designated here by 21.5, 26.6 and 38 per cents of ammonium sulphate in the precipitated mixture. They actually mean a content of 2.9, 3.33, and 4.9 c.c. of saturated ammonium sulphate solution in 10 c.c. of the precipitated mixtures, which would then contain, respectively, 15.67, 18.00 and 26.50 gms. of the dry ammonium sulphate per 100 c.c.—figures which by no means or method of interpretation can be logically expressed by 21.5, 25-6 and 38 percentages of ammonium sulphate. Fortunately the fault lies in the nomenclature only, the precipitations being accomplished by the use of saturated ammonium sulphate solution.

Twenty liters of plasma (475 units per c,c.), were diluted with 20 liters of water, by fractioning with saturated ammonium sulphate solution, the three proteid precipitates were obtained which separated at concentrations corresponding to 3.3 c.c., 3.3—3.8 c.c. and 3.8—5.0 c.c. of the saturated salt solution in 10 c.c. The saturated sodium chloride soluble antitoxic) globulins of these fractions and of the 5.0 saturation precipitate of a second 20 liters of the plasma were prepared as usual. Proteid determinations (coagulations) and potency tests were duplicated.

Prep. 77.

Fractions.	A. 0.0-5.0	B. 0.0-3.3	C. 3.3-3.8	D. 3.8-5.0
Volume c.c.	5,200	1,440	1,400	2,050
Units per c.c.	1,450	1,150	1,350	1,750
Times concentrated	3.05	2.42	2.84	3.68
Per cent recovered	79.3	17.4	19.9	37.8
Proteid, gms. per 100 c.c.	11.66	11.51	9.87	9.70
Units, per gm. proteid	12,436	10,000	13,666	18,000

A second experiment with a 450 unit plasma gave the following results:

Prep. 82.

Fractions.	A. 0.0-5.0	B. 0.0-3.3	C. 3.3-3.8	D. 3.8-5.0
Volume c.c.	6,240	1,350	1,640	2,550
Uuits per c.c.	1,050	900	1,300	1,600
Times concentrated	2.34	2.00	2.89	3.56
Per cent recovered	72.8	13.9	22.6	45.3
Proteid. gms. per 100 c.c.	10.59	12.06	13.46	13.41
Units, per gm. proteid	9,914	7,464	9,655	11,930

These observations show that the antitoxic globulins of the higher fraction are much more potent than those of the less soluble proteids.

Both the preparations by the half-saturation ammonium sulphate method and by fractioning, when precipitated from the saturated sodium chloride solution and dialyzed, contained a probably partially denaturalized antitoxic globulin: this had a diminished solubility and antitoxic potency (per gram proteid) and was precipitated on slight acidification by diluting twenty times. The filtrates from the water-acid precipitable globulin coagulated at 73 degrees, while saline solutions of the precipitates so obtained showed varying and much lower coagulating temperatures. The solutions of the high proteid fractions have a peculiar green color. A redetermination of the precipitation limits of the globulin in the three fractions after removal of the water-acid precipitable proteid, showed that the different precipitation limits were relatively characteristic for the fractions.

The following results were obtained on progressively fractioning (in two experiments) by the addition of the dry salt[1] to a liter of about 400 units antitoxic plasma. The initial dilution was 1:5. The precipitates were pressed between filters and extracted with saturated sodium chloride solution. The determinations on the filtered extracts are given per c.c. of the original plasma. The results are roughly quantitative only, loss of the filtrate in pressing out the precipitated globulins being disregarded. Proteid determinations and potency tests were duplicated.

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[1] Calculations or reference tables for the amounts of salt to be added to produce or raise a proteid solution to any desired concentration may accurately be made by employing the following formula:

$$X=\frac{v\,p\,(c_2-c_2)}{10-e\,p\,c_2}$$ where x is the number of gms. of salt to be added to give the required concentration, v the original volume in c.c., e the increase in the volume of the solvent by 1 gm. of salt, p the gms. of salt per c.c. of its saturated solution, and c_1 and c_2 are the initial and desired degrees of saturation, expressed as c.c. in 10 c.c. For $(NH_2)_2SO_4$ e and p may be regarded as approximately 0.54; then

$$X=\frac{v\,(c_2-c_1)}{18.158-0.54\,c_2}\text{ ; and when } c_1=0,\ X=\frac{v\,c_2}{18.158-0.54\,c_2}$$

To raise the concentration by the addition of saturated salt solution the amounts (c.c.) of the original proteid solution and of the saturated salt solution in the mixture are calculated; also the amount of the salt solution necessary to bring the *proteid solution* to the desired concentration. Sufficient excess of saturated salt solution over that already present is added to make the required total.

Fractioning of Plasma 305, 8/1/06.

Fractions.	Proteid per c.c.	Units per c.c.	Units per Gram of Proteid.
A			
0.0–3.4	0.00321	25	[illegible]
3.4–3.6	0.00223	20	[illegible]
3.6–3.8	0.00432	47	[illegible]
3.8–4.0	0.00416	52	12.500
4.0–4.2	0.00408	60	14.705
4.2–4.4	0.00272	55	20.220
4.4–4.6	0.00191	40	[illegible]
4.6–4.8	0.00163	32	19.632
4.8–5.0	0.00111	[illegible]	[illegible]
5.0–5.6	0.00428	18	4.205
B			
0.0–3.4	0.00394	27	[illegible]
3.4–3.6	0.00219	20	[illegible]
3.6–3.8	0.00397	45	11.335
3.8–4.0	0.00336	50	14.880
4.0–4.2	0.00332	60	18.072
4.2–4.4	0.00225	55	21.568
4.4–4.6	0.00181	[illegible]	22.094
4.6–4.8	0.00147	30	20.408
4.8–5.0	0.00093	18	[illegible]
5.0–5.6	....	18	..

In each instance there is a progressive increase in potency as the antitoxic globulin becomes more soluble in the fractions until a concentration of the salt of about 4.2 is reached. The potency per gram remains then practically constant at about three times that of the saturated sodium chloride extract of the euglobulin fraction (0.0—3.4) until between 4.8 and 5.0 saturation; above this limit the potency per gram rapidly diminishes to a relatively very low figure. Between the 4.2 and 4.8 limits, over half the units of the original plasma are precipitated, while the antitoxin is contained in less than one-third of the total antitoxic globulin.

The fact that the major portion of the antitoxin remained soluble at a concentration of 4.2 saturation, led us to investigate whether the protective material was mechanically precipitated with the proteid of the lower fractions. Such a result seemed *a priori* improbable because the individual fractions were at such frequent intervals and contained such a small amount of the globulin precipitate as to make hardly conceivable a mechanical inclusion of more soluble colloidal particles for more than a few minutes' duration. Our plan was to fraction the antitoxic plasma at 4.2 saturation. The lower fraction (precipitate) was to be dissolved in an added known volume of water, reprecipitated at 4.2 saturation and after standing 24-48 hours was to be filtered. The procedure was twice repeated with the precipitates obtained at 4.2 saturation. The three filtrates and the saturated sodium chloride soluble (antitoxic) globulin of the final 4.2 saturation precipitate were examined for globulin and antitoxic content. We were not able to separate by three times repeated fractioning at 4.2, the antitoxin from the lower fraction; over half the antitoxin brought down at first was pronouncedly a constituent of the precipitate, the amount in the filtrate from the final precipitation being very slight (though the potency per gram of proteid was relatively high). The protocol follows:

250 c.c. of antitoxic plasma (305, 8/10/06, 300+units per c.c.) were diluted with 475 c.c. of water and precipitated with 525 c.c. of saturated ammonium sulphate solution. After standing 24 hours, the precipitated globulin was filtered off. To the filtrate, 1000 c.c., was added 60 gms. of dry ammonium sulphate after sufficient ammonium sulphate solution had been employed to give 1500 c.c. at half saturation. The resulting precipitate (4.2–5.5 saturation) was pressed out between filters, dissolved and made up to 200 c.c.

The precipitate at 4.2 saturation was pressed out between filter paper, dissolved by the addition of 580 c.c. of water and reprecipitated with 420 c.c. of saturated ammonium sulphate solution. The total volume of the precipitated mixture was slightly over 1000 c.c. After standing 24 hours, the reprecipitated globulin was filtered off from "Filtrate I" (900 c.c.).

The precipitate from I was dissolved in 580 c.c. of water and precipitated with 420 c.c. of saturated ammonium sulphate. Filtrate II was 930 c.c.

Filtrate III similarly obtained amounted to 950 c.c. The globulin precipitate was extracted with 1000 c.c. of saturated NaCl solution.

Determinations of proteid and antitoxic content are given per c.c. of the original plasma.

	Proteid per c.c.	Units per c.c.	Units per Gram of Proteid.
Filtrate I	0.00270	40	14.810
" II	0.00332	50	15.080
" III	0.00075	12	16,100
4.2 Ppt. gbl. (ext. NaCl)	0.01066	125	12,100
4.2—5.6	0.00685	80	11.680
	0.02428	307	

Further fractioning after complete removal of the water precipitable globulin was done on 50 c.c. of the globulin solution, Prep. 77A (cf. p. 255). The fractioning was made at a dilution of the original preparation of 1:20. The results are expressed per c.c. of the original undiluted preparation.

Refractionation of Preparation 77a.

Fraction.	Proteid per c.c.	Units per c.c.	Units per Gram of Proteid.
0.0—4.0	0.0408	400	9.791
4.0—4.4	0.0165	225	13,667
4.4—5.0	0.0176	375	21,306
5.0+	0.0018	75	41,722
4.8—5.5*	0.0046	150	34.783

* Made on a second 50 c.c. of the same preparation.

The refractioning of 77A from which the water-acid precipitable globulin had been removed, showed a marked progressive increase in potency hand in hand with the greater solubility of the proteid.

Fraction 3.8—5.0 of Prep. 82 was refractioned without removing the water-acid precipitable globulin. The dilution was 1:10.

Refractionation of Preparation 82d. (High Potency Fraction.)

Fraction.	Proteid per c.c.	Units per c.c.	Units per Gram of Proteid.
0.0—4.0	0.07318	600	8.136
4.0—4.2	0.91779	240	13.490
4.2—4.4	0.02197	260	11.840
4.4—4.6	0.01232	160	12.990
4.6—4.8	0.00708	90	12.711
4.8—5.0	0.00511	80	15.670
5.0—5.6	0.00197	90	45.690
	0.13941	1,510	
For 82D.	0.1341	1,600	

82D contained a globulin of rather uniform potency per gram from fractions 4.0—4.8; then a marked jump for the fraction 5.0—5.6 to about three times the original potency per gram was observed. The portion of antitoxin in the highest fractions of 77A and 82D was less than 6 per cent. of the total units. Prepared for administration as is the ordinary antitoxic globulin, the resulting product would have had a potency of from 5000—6000 units per c.c.

The high antitoxic potency per gram proteid of the globulin in the preparations precipitable between 4.8 and 5.6 saturation led us to attempt to obtain such a product in bulk from the antitoxic globulin preparations (Gibson) and directly from plasma. Our experiences have comprised (1) the influence of the reaction of the plasma, (2) repeated extraction (with 4.8 saturation ammonium sulphate) of the globulins to dissolve out the mechanically precipitated highly potent antitoxic substances, and finally (3) progressive denaturalization of the globulin by repeated extractions with saturated sodium chloride solution and reprecipitation with the sulphate. Our results, however, have not been encouraging. The protocols are given below:

(1) *a.* 250 c.c. of antitoxin plasma (262, 10/22/6, 500 units per c.c.), were diluted with 1050 c.c. distilled water and precipitated at 4.8 with 1200 c.c. of saturated ammonium sulphate solution. After standing three hours it was filtered and the filtrate (2250 c.c.) raised to 5.0 saturation by adding 28.6 gms. of dry $(NH_4)_2SO_4$. After 24 hours at room temperature, the half saturation precipitate was filtered off, pressed between filters and made up to 225 c.c. Of the 5.0 saturation filtrate, 2200 c.c. were precipitated at 5.6 saturation with 96 gms.

of dry $(NH_4)_2SO_4$ and filtered after 24 hours' standing at room temperature. The precipitate was pressed out and made up to 218.5 c.c. in distilled water.

b 250 c.c. of the same plasma were made distinctly alkaline with $\frac{N}{10}$ NaOH and then fractioned exactly as in *a*.

c. 250 c.c. of the plasma were made distinctly acid with dilute acetic acid, and then similarly fractioned.

Proteid coagulations and potency tests were made as usual.

Plasma.	Fractions.	Proteid per c.c.	Units per c.c.	Units per Grams of Proteid.
a. Native............	4.8–5.0	0.0015	11	7.333
	5.0–5.6	0.0051	20	3,921
b. Alkaline...........	4.8–5.0	0.0011	11	10,000
	5.0–5.6	0.0947	20	4.255
c. Acid...............	4.8–5.0	0.0012	11	9.175
	5.0–5.6	0.0060	24	4,000

(2) 500 c.c. of the plasma of the bleeding employed for reprecipitation at 4.2 (305, 8/10/06, 300 units per c.c.)[1] were diluted with 800 c.c. of water and precipitated at 4.8 saturation with 1200 c.c. of saturated ammonium sulphate solution. After standing for 24 hours, the precipitate was filtered off. Of the filtrate, 2250 c.c. were precipitated, at about 5.6 saturation, with 116 gms. of dry $(NH_4)_2SO_4$; after standing, the precipitate was separated, pressed out between filter paper and made up in solution to 450 c.c. with water (fraction 4.8–5.6). The moist precipitate obtained at 4.8 saturation was thoroughly suspended in about 1500 c.c. of 4.8 saturation $(NH_4)_2SO_4$ and filtered after standing for two days, during which time the mixture was occasionally shaken up. The precipitate from the first filtrate (I) was re-extracted as before, this procedure being carried on, in all, four times. The precipitate then remaining was made up with saturated NaCl solution to a volume of 1000 c.c. Proteid determinations and the antitoxin tests were made on the fraction 4.8–5.6, on the four filtrates and on the NaCl extract of the residue, and are tabulated as before per c.c. of the original plasma.

	Proteid per c.c.	Units per c.c.	Units per Gram of Proteid.
4.8-5.6 sat..............................	0.00191	12*	6.316
Filtrate I..........................	0.00240	10*	4.170
" II..........................	0.00160	9*	15,000
" III..........................	0.00026	8*	30.770
" IV..........................	0.00030	4*	13.333
Residue..................................	0.01784	250	14.020
	0.02431	293	

* Because of the low antitoxic and high (toxic) $(NH_4)_2SO_4$ content, the tests were made with 25 or 50 m.l.d. instead of the 100 m.l.d. ordinarily employed.

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[2] Cf. pp. 101 and 102.

The amount of antitoxin and of proteid in the filtrates was so small that slight errors in the determinations would influence greatly the calculations of the antitoxin units per gram of proteid. Yet the results obtained on Filtrate III., when the figures on the preparations are recalled (p. 259), make it highly probable that a very small portion of the antitoxin can be separated in a much more highly potent form than is the case for the bulk of this substance.

Progressive denaturalization of the proteid as a means of separating the globulin from the antitoxic substance, if other than the serum globulin itself, has not proved successful. The method used was to extract the ammonium sulphate globulin precipitates of plasma or the antitoxic globulin preparation with saturated sodium chloride solution, to filter off the insoluble globulin residue (after some days standing), and to reprecipitate the filtrate by the addition of a little over half its volume of saturated ammonium sulphate solution. The extraction of this last precipitate with the sodium chloride and the precipitation of the filtrate with ammonium sulphate followed. This procedure was carried on 4—6 times on the 4.8 residue of (2), and on two antitoxic globulin solutions, which were obtained by the sodium chloride extraction of 3.8 saturation precipitates (less potent fraction), one of which was already thoroughly denaturalized in preparation because of the accidental partial desiccation of the acidified saturated sodium chloride precipitated proteid before dialysis. The final filtrates of one of the sodium chloride extracts of the originally denaturalized antitoxic globulin preparations had a potency of almost 15,000 units per gram of proteid. The globulin solution of the 4.8 saturation extraction precipitate and the second antitoxic globulin preparation contained about 10,000 units per gram of proteid.

The injection of the antitoxic globulins of the various globulin preparations sensitizes guinea pigs to subsequent, otherwise non-fatal intraperitoneal administration of serum (Smith and Rosenau and Anderson). Injected intraperitoneally into sensitized guinea pigs, the typical convulsions produced by serum are incited, and the deaths of the animals may ensue. Rashes of the urticarial character with little or no accompanying constitutional symptoms may follow the therapeutic administration of the several fractionally precipitated antitoxic globulins.

Therapeutically there is no difference in the results obtained with the equivalent unit injections of either the high (3.8 + saturation) or low (3.3 saturation), fractions of preparations 77 and 82 (pp. 255 and 256).

Conclusion.

From the data presented, it appears that the *saturated sodium chloride soluble* serum globulins of the higher fractions are uniformly much more potent per gram of proteid in antitoxin than are those precipitated by lower concentrations of ammonium sulphate. Between concentrations of the sulphate of 5.0 and 5.6, a small proportion of the total sodium chloride soluble globulin of the antitoxic globulin preparation (Gibson) or of a higher fraction of the same is precipitated; the solution of this globulin has a protective power of over 40,000 units per gram of proteid. The direct fractioning of the plasma, however, does not yield so potent a product; at a dilution of 1:5 of a 400 unit plasma the globulin remaining in solution at 4.2 and precipitated at 4.8 saturation has a potency of about 20,000 units per grain of proteid. It is thus practicable to prepare an antitoxic solution of over 2,000 units per c.c. from a relatively low plasma.

Whether or not this difference in the potency per gram of proteid is associated with the presence of non-antitoxic globulins having the same fractional precipitation limits as the protective substance remains as yet undecided. It is possible that such a variation in potency may be purely physical, associated with the size or condition of aggregation of the colloidal globulin particles—the less soluble larger masses having diminished antitoxic properties. Certainly, however, we find the antitoxin is characterized by a wide range of the precipitation limits similar to the soluble globulins, *i. e.*, in spite of repeated precipitations, a part of the antitoxin is comparatively insoluble in concentrations of ammonium sulphate in which the major portion of the protective substance readily dissolves.

In concluding the present paper, we desire to express our appreciation of Dr. Park's suggestions and helpful criticism.

ON THE FRACTIONATION OF AGGLUTININS AND ANTITOXIN.

By Drs. Robert Banks Gibson and Katharine R. Collins.

E. P. Pick,[1] in 1901, associated a number of antisubstances individually with the one or the other of the two serum globulin fractions of the Hofmeister classification. In the pseudoglobulin (3.4 to 4.6 saturated ammonium sulphate solution)[2] group of antibodies he placed the diphtheria and tetanus antitoxins and the typhoid agglutinin of horse serum; the lower or euglobulin fraction (2.9 to 3.4 saturated), comprises diphtheria and tetanus antitoxin and cholera lysin in the goat, rabbit and guinea pig, and finally cholera agglutinin in the horse and goat. It becomes possible, according to Pick, to separate the individual specifically reacting antisubstances by fractioning appropriate mixtures of sera. Such a possibility suggested the application of this method to the further study of certain antibodies, especially of the relation of specific and group agglutinins developed by immunization against a single strain of organism. Preliminary experiments in the course of our investigation indicated the unreliability of Pick's differentiation, and attention was accordingly directed to the actual possibility and practicability of distinguishing between antibodies by fractionation of the globulin. The availability of polyagglutinative sera for the work gave a chance for making numerous and extended observations of the distribution of these antibodies in the fractions.[3]

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[1] *Beitr. z. chem. Physiol. u. Path.*, i, p. 351, 1901.

[2] The degrees of saturation, as here expressed, indicate a concentration equivalent to a content in 10 c.c. of the precipitated solution of 3.4 and 4.6 c.c. of saturated ammonium solution respectively.

[3] A preliminary account of our results was published several months ago in the *Proceedings of the Society for Experimental Biology and Medicine*, iv, p. 15, 1906–1907.

The literature on the fractional precipitation of the antibodies is not extensive. Ide and Lemaire[1] (in 1899), found the precipitation limits of diphtheria antitoxin in horse serum to be from 2.8—4.4 saturation. Fuld and Spiro[2] (1900), associated the antirennin of horse serum with the pseudoglobulin, while a milk-coagulating action was possessed by the eu-fraction. The failures of Brodie and of Atkinson (of this laboratory), to separate diphtheria antitoxin from the accompanying serum globulins are referred to in the following paper. Porges and Spiro,[3] without giving any of their experimental protocols divide, according to the distribution of the antibodies, the serum globulin into three distinct fractions; the ammonium sulphate precipitation boundaries of these overlap unless the serum is greatly diluted. Landsteiner[4] found that the antitryptic action of blood serum is possessed by the albumin precipitated by complete saturation with ammonium sulphate after removal of the globulin. Cathcart[5] also observed the antitrypsin to be associated with the albumin but not with the globulin fraction. Glaessner[6] states that the euglobulin fraction inhibits the action of trypsin, but the typical protocol which he publishes and his statement of the Hofmeister classification show a misconception and confusion of the identity of his fractions.[7] Glaessner apparently found that the globulin remaining in solution on dialysis was antitryptic.

Very recently Simon, Lamar and Bispham[8] found that the opsonic substance in blood serum was precipitated with the serum proteids which separated out on dialysis. Opie and Barker[1] observed that proteolysis in an alkaline medium by the enzyme of the leucocytes is inhibited by the serum albumin.

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[1] Ide and Lemaire: *Arch. internat. d. pharmacodyn.*, vi, p. 477, 1899.

[2] Fuld and Spiro: *Zeitschr. f. physiol. Chem.*, xxxi, p. 133, 1900.

[3] Porges and Spiro: *Beitr. z. chem. Physiol. u. Path.*, iii, p. 277, 1903.

[4] Landsteiner: *Centralbl. f. Bakt.*, xxvii, Abt. I, p. 357, 1900.

[5] Cathcart: *Journ. of Physiol.*, xxxi, p. 497, 1904.

[6] Glaessner: *Beitr. z. chem. Physiol. u. Path.*, iv, p. 79, 1904.

[7] The error on his part has not heretofore been noted. Glaessner states (p. 82): "Das Globulin des Blutserums lässt sich nach den in Hofmeisters Laboratorium in mindestens 3 Fractionen zerlegen: in das bei 25 Proz. Sättigung mit Ammonsulfat ausfallbare Fibrino-gloubulin, in das Euglobulin, das bei einer Sättigung von 33 Proz. ausfällt und bei der Dialyse in Lösung bleibt, und endlich in das bei 38 Proz. Sättigung ausfallbare bei der Dialyse unlösliche Pseudoglobulin." The confused nomenclature of the degrees of salt saturation is discussed in the preceding paper (p. 98).

[8] Simon, Lamar and Bispham: *Journ. of Exp. Med.*, viii, p. 651, 1906.

In a paper just published on the relation of diphtheria antitoxin to the serum globulin, Ledingham[2] finds that the pseudo-globulin of horse serum contains the greater part of the antitoxin. The repeatedly precipitated euglobulin, however, in one horse still contained fully 10 per cent. of the antitoxin; in a second horse, the euglobulin similarly treated contained practically none of the antitoxin. Single precipitations (without further purification) showed that large amounts of antitoxin may be carried down with the lower fraction; with the one horse, judging in part from the tests on the pseudoglobulin fraction, over half the units must have precipitated with the euglobulin.[3] Ledingham also confirm our own observation here reported that the diphtheria antitoxin of the goat is not invariably linked to the euglobulin fraction as maintained by Pick.

Recently observations have been made by Ruediger[4] on the relation to the blood proteids of streptolysin, the hemolytic substance produced by the development of streptococci in heated serum. The lysin was precipitated with the globulin by saturation with magnesium sulphate; it was found with both the euglobulin and pseudoglobulin of the fractioned undiluted serum and also in both the insoluble proteid and the filtrate of the dialyzed half saturation ammonium sulphate precipitate.

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[1] Opie and Barker: *Journ. of Exp. Med.*, ix, p. 207, 1907.

[2] Ledingham: *Journ. of Hygiene,* vii, p. 65, 1907.

[3] Brieger (*Festschrift fü R. Koch,* Jena, 1903) and also one of us (Gibson) have already reported similar experiences. In some as yet unpublished experiments carried on for another purpose by one of us (Gibson) and E. J. Banzhaf of this laboratory, it has been found that if undiluted horse serum be precipitated with half its volume of saturated ammonium sulphate solution and allowed to stand for 18 hours, the euglobulin precipitate may contain over two-thirds of the total serum globulin. Precipitation under the same conditions except that the precipitated mixture is 5 or 10 times the volume of the original serum gives a euglobulin figure only of from a fifth to a third the total globulin. The euglobulin at a dilution of 1:10 is noticeably smaller than at 1:5. This fact explains the diminished antitoxic content of reprecipitated or washed euglobulin fraction and makes difficult any hard and fast division into eu- and pseudo-globulins.

[4] Ruediger: *Journ. of Infect. Diseases,* iv, p. 377, 1907.

Moll,[1] however, has shown that such heating suffices to alter the chemical composition and to change the precipitation characters of the blood proteids.

Owing to the difficulty of making a hard and fast division of the serum globuline into the eu- and pseudo-fractions, our experiments were not planned to be interpreted especially from the quantitative occurrence of the agglutinins in the one or the other fraction of specific agglutinating sera. We have aimed, however, to determine if the relative proportion of the agglutinins of polyagglutinative sera in the fractions remained constant as regards the proportional distribution of all the agglutinins of the serum in the eu- and pseudo-globulins. In a word, by a difference of precipitation limits to ammonium sulphate, it should be expected that the bulk of one or more of the agglutinins would appear in the one fraction, as contrasted with the larger proportion of each of the remaining agglutinins occurring in the other fraction. Attention is particularly directed in studying the results from our standpoint to the pseudoglobulin fraction (filtrate from the euglobulin fraction). Any loss from the euglobulin fraction through solubility in the wash solution has been considered only in the case of the antitoxins. Such loss may be interpreted as due to the resolution of the mechanically precipitated proteids of the more soluble fraction; it may be considered just as well in part as a not absolute insolubility of the eu- fraction in 3.4 saturation ammonium sulphate. The content of agglutinins in the washed euglobulin fraction is of interest, however, as it is more highly "purified" than the pseudoglobulin, so that any relative differences in the distribution of the agglutinins should be from this standpoint the more pronounced for the low fraction.[2] The limitations in determining the agglutinative potency of the serum and of the fractions, however, make difficult at times the interpretation of the readings obtained, and do not permit conclusions being drawn from a single experiment.

It was found repeatedly in our experiments with rabbit and goat sera that the agglutinins for the dystentery group of organisms (Flexner Manila and Shiga), typhoid, colon and cholera, were not confined to either the pseudoglobulin of the washed (with 3.4 saturated ammonium sulphate solution) euglobulin fractions; they were either split by the fractioning, the larger portion occurring in the pseudoglobulin, or almost the entire amount of the agglutinating substances recovered were in this higher fraction in the original quantitative proportion to one another. With antidysentery horse serum, the dysentery (Shiga and Flexner) and *B. coli* agglutinins were fairly quantitatively split between the pseudo- and euglobulin fractions, the latter containing the lesser amount. With an anticholera and anti-typhoid horse serum, the pseudoglobulin (two experiments) and also the filtrates from two additional 3.6 and 3.8 saturation precipitations contained the bulk of both the agglutinins. In subsequent experiments with sera from other bleedings as well as with the sera used above, the typhoid agglutinin was divided between the two fractions with a somewhat larger proportion occurring in the pseudoglobulin.

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[1] Moll: *Beitr. z. chem. Physiol. u. Path.*, iv, p. 563, 1904.

[2] The euglobulin of 2 c.c. of serum precipitated at a final dilution of 1:5 was washed usually by being three times thoroughly suspended in 10 c.c. of 3.4 saturation ammonium sulphate solution and recentrifuged.

The results of exhaustion experiments on the two globulin fractions were the same as those that would be obtained in the use of the native serum, and failed to give any reason for believing that we were dealing with a separation of group and specific agglutinins through fractioning.[1]

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[1] Immunization with certain bacteria results in the development of "group" or "common" and of "specific" agglutinins in the serum of the animal immunized. Group agglutinins are agglutinating substances which are effective both on the homologous organism and on some allied strains of bacteria; specific agglutinin is effective on the organism used for immunization. An immune serum developed by immunization against *B. dysenteriæ* (Shiga) might contain, for the sake of illustration, simply (1) a group agglutinin effective for Shiga, Flexner Manila, Pfeiffer and *B. coli*, and (2) an agglutinin specific for the Shiga strain. When immunization has been developed simultaneously against two or three of the above organisms instead of the Shiga strain alone, the number and agglutinating scope of the agglutinins resulting becomes more complex, various group agglutinins and the specific agglutinins for each organism being present. The existence of the two types of agglutinins is demonstrated by the agglutinating characters of the diluted serum after the agglutinins for any desired strain of organism have been exhausted by adding suspensions of washed organisms; after contact for some hours the agglutinated and excess of free organisms are removed by filtration. The agglutinins for several related strains can thus be successively withdrawn. In the example given above, exhaustion with either *B. coli*, Flexner Manilla or Pfeiffer would remove only the group agglutinin (1) so that the serum would still agglutinate the Shiga, though at a diminished dilution; exhaustion with the Shiga would take out both the group and the specific agglutinins, and this serum would no longer have agglutinating properties for any of the above organisms. Cf. Castellani: *Zeitschr. f. Hyg. u. Inf.*, xl, p. 1, 1902; also Park and Collins: *Journ. Med. Research*, xii, p. 491, 1904.

Precipitation of antidiphtheria goat serum (three experiments) showed that less than half the antitoxin remained in the pseudoglobulin; practically none was found in the euglobulin while the 3.4 saturated ammonium sulphate solution washings contained the larger part. Our results with the antitoxic horse serum at a dilution of 1:5 are essentially identical with Pick's.

The results of the work accomplished have demonstrated the untrustworthiness of any such differentiation of the antibodies as those contained in the euglobulin and those of the pseudoglobulin. No evidence has been adduced from our experiments to show that the agglutinins developed in the rabbit, goat and horse can be classed as belonging to either globulin, or that these antibodies can be separated from one another by ammonium sulphate fractioning of polyagglutinative sera.[1]

A description of the experimental procedure is given with the protocols which follow:

Fractionation of Polyagglutinative Rabbit Serum.

Combined immunization against Flexner Manila dystentery, Shiga dystentery, Pfeiffer,[2] colon and cholera. Rabbit bled II/27/06. Serum fractioned II/29/06.

Five c.c. of the rabbit serum diluted with 11.5 c.c. of distilled water, were precipitated by 8.5 c.c. of saturated ammonium sulphate solution. After standing two hours, 12.5 c.c. of the uniform mixture were removed and centrifuged in stoppered tubes. The supernatant fluid contains the "pseudoglobulin" fraction. The precipitate ("euglobulin") was washed three times by being thoroughly suspended in 3.4 saturated solution and centrifuged; it was once more suspended and the volume made up to 12.5 c.c. The agglutinating properties were then ascertained of (1) the original serum; (2) the total globulin, a uniform sample of the precipitated serum; (3) the pseudoglobulin fraction, and (4) the washed 3.4 saturation precipitate or euglobulin fraction. Agglutinations were determined microscopically and control slides were examined. Dilutions are in terms corresponding to the original serum. The characters of the agglutinations at the various dilutions are indicated as follows:

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[1] In the paper by Banzhaf and Gibson following this article, it will be shown that the globulins of the serum do differ markedly in their content of antitoxin per gram proteid.

[2] The original Pfeiffer strain of *B. typhosus*.

+ + + +	agglutination with no free organisms.
+ + +	agglutination with relatively very few free organisms.
+ +	agglutination but with numerous free organisms.
+	incomplete agglutination, small loose groups and many free bacteria.
±	tendency to agglutinate.
–	no aggltunation.
°	observation lost.

The procedure was essentially unchanged in the other experiments. The serum used in this and the following experiments was roughly tested for orientation before the final agglutinations were made. A **+++** agglutination indicates usually the highest dilution for an observed position reaction. It should be remembered that the character of the agglutination at any dilution is often difficult to decisively determine; *the actual observations are by no means so exact as would be inferred from the published experiments of Pick and of others.*

The agglutinating properties of this rabbit serum (Table I.), were too low to be entirely satisfactory for fractioning, the weakest agglutinating action (1:50) being manifested on the Shiga strain. The agglutinin for this organism drops out in the euglobulin fraction. This lost agglutinin is not found in the pseudo fraction. It is conceivable from this experiment that the agglutinin of the Shiga dysentery is more soluble than that of the other five strains; however, the Shiga shows no such differences in the two following precipitation experiments on later bleedings of the same rabbit. It is more likely that the content of serum in agglutinin was originally so low that in the fractioned and washed euglobulin the dilution of 1:20 failed to show the proportion of agglutinin present. With the Flexner Manila dysentery, the Pfeiffer typhoid strain, the colon and the cholera—all contained in greater concentration than the Shiga agglutinin—the major portions of the agglutinins occur in the pseudoglobulin, a smaller amount being held by the low fraction.

TABLE I.

Fractioning of Polyagglutinative Rabbit Serum.

Bleeding of II/27/06; fractioning II/29/06.

Organism.	Fraction.	20	50	100	200	500	1000
Flexner Manila.....	Original serum	++++	++++	++++	**++++**	++	±
	Total gbl.	++++	++++	++++	**++++**	++	—
	Pseudogbl.	++++	++++	**++++**	+	±	—
	Eugbl.*	++++	++++	**++++**	±	—	—
Shiga...............	Serum	++++	**++++**	++	±	—	—
	Total gbl.	++++	**++++**	+	—	—	—
	Pseudogbl.	**++++**	±	—	—	—	—
	Eugbl.*	—	—	—	—	—	—
Pfeiffer.............	Serum	++++	++++	**++++**	++	±	—
	Total gbl.	++++	++++	**++++**	++	—	—
	Pseudogbl.	++++	**++++**	+	±	—	—
	Eugbl.*	++++	**++++**	+	—	—	—
Colon...............	Serum	++++	++++	++++	**++++**	±	—
	Total gbl.	++++	++++	**++++**	++	±	—
	Pseudogbl.	++++	++++	**++++**	+	±	—
	Eugbl.*	++++	**++++**	+	—	—	—
Cholera.............	Serum	++++	++++	++++	**++++**	±	—
	Total gbl.	++++	++++	++++	**++++**	±	—
	Pseudogbl.	++++	++++	**++++**	±	—	—
	Eugbl.*	++++	**++++**	±	—	—	—

* Tested III/1/06 with a fresh culture.

Exhaustion of the two globulin fractions (Table II.) with suspensions of the Flexner Manila strain has not withdrawn the agglutinins for the typhoid from either fraction. Exhaustion with the Pfeiffer likewise is not complete for the dysentery in either pseudo- or euglobulin.

This later bleeding of the same rabbit shows by far the greater part of all the agglutinins in the pseudoglobulin (Table III.), and small though relatively proportional amounts of each in the low fraction. The absolute dropping out of the Shiga does not occur as in the preceding fractioning. The results are also more uniform.

TABLE II.

Exhaustion Experiment, Polyagglutinative Rabbit.

Exhaustion of the Original Serum and the Fractions (cf. Table I.).

Exhaustion with Flexner Manila.

Organism.	Fraction.	20	50
Flexner Manila........	Serum	+	—
	Pseudogbl.	—	—
	Eugbl.	—	—
Pfeiffer........	Serum	++++	++++
	Pseudogbl.	++++	++++
	Eugbl.	++++	++++

Exhaustion with Pfeiffer.

Flexner Manila........	Pseudogbl.	++++	—
	Eubgl.	++++	—
Pfeiffer........	Pseudogbl.	—	—
	Eugbl.	—	—

TABLE III.

Fractioning of Polyagglutinative Rabbit Serum.

Serum from bleeding IV/16/06; serum fractioned IV/18/06.

Organism.	Fraction.	50	100	200	500	1000	2000
Flexner Manila.....	Total gbl.	++++	++++	++++	++++	**+++**	+
	Pseudogbl.	++++	++++	++++	**++++**	++	+
	Eugbl.	++	+	—	—	—	—
Shiga........	Total gbl.	++++	++++	++++	++++	**+++**	++
	Pseudogbl.	++++	++++	++++	**++++**	++	±
	Eugbl.	++	+	—	—	—	—
Cholera	Total gbl.	++++	++++	++++	++++	**++++**	++
	Pseudogbl.	++++	++++	++++	++++	**++++**	+
	Eugbl.	+	—	—	—	—	—
Pfeiffer........	Total gbl.	++++	++++	++++	**++++**	++	+
	Pseudogbl.	++++	++++	++++	**++++**	+	±
	Eugbl.	±	—	—	—	—	—

The third precipitation (Table IV.), shows apparently a recovery of all the agglutinins in the high globulin fraction. The pseudoglobulin dilutions for the Flexner Manila, in fact, were read slightly higher than were those of the total globulin.

Pick's single observation by the test-tube method on typhoid rabbit serum, agglutinating at 1:20,000, gave the limit of agglutination of the pseudoglobulin at 1:3000, of the euglobulin 1:20,000; after reprecipitating the fractions three and four times, respectively, the limits of dilution for agglutination were at 1:20 and 1:8000.

Table IV.

Fractioning of Polyagglutinative Rabbit Serum.

Serum from bleeding on V/23/06.

Organism.	Fraction.	50	100	200	500	1000
Flexner Manila	Total gbl.	++++	++++	++++	++++	**+++**
	Pseudogbl.	++++	++++	++++	++++	**++++**
	Eugbl.	—	—	—	—	—
Pfeiffer	Total gbl.	++++	++++	++++	++++	**+++**
	Pseudogbl.	++++	++++	++++	++++	**+++**
	Eugbl.	—	—	—	—	—
Cholera	Total gbl.	++++	++++	++++	**+++**	++
	Pseudogbl.	++++	++++	++++	**+++**	++
	Eugbl.	±	±	±	—	—

Polyagglutinative Goat Serum.

Immunization against Flexner Manila, Shiga, Pfeiffer, cholera and *B. coli.*

The proportion of the agglutinins for each organism is much greater in the pseudoglobulin than in the euglobulin fractions (Table V.). This conclusion is confirmed for the Shiga, Pfeiffer and cholera by the redetermination of the agglutinations in the exhaustion experiment (Table VI.). The Flexner Manila dysentery strain has not exhausted the agglutinins for the other organisms completely from the eu- or from the pseudoglobulin fractions; nor have the agglutinins apparently been withdrawn to a relatively greater degree from the one than from the other fraction.

Fractioning of the polyagglutinative serum (of lessened agglutinating power) from the second bleeding of the goat immunized against the mixed cultures showed that the agglutinins were almost quantitatively contained in the pseudoglobulin fraction (Table VII.).

TABLE V.

Fractioning of Polyagglutinative Goat Serum.

Serum from bleeding II/27/06; fractioned III/9/06.

Organism.	Fraction.	50	100	200	500	1000
Flexner Manila............	Serum	++++	++++	++++	**+++**	++
	Total gbl.	++++	++++	++++	**+++**	+
	Pseudogbl.	++++	++++	**+++**	++	–
	Eugbl.	++++	**+++**	–		
Shiga......................	Serum	++++	++++	++++	**++++**	++
	Total gbl.	++++	++++	++++	**++++**	+
	Pseudogbl.	++++	++++	++++	**++++**	–
	Eugbl.	++++	**+++**	–		
Pfeiffer.....................	Serum	++++	++++	++	++	+
	Total gbl.	++++	++++	+++	**++++**	±
	Pseudogbl.	++++	**++++**	++	+	±
	Eugbl.	**++++**	+	–		
Cholera........	Serum	++++	++++	++++	++++	**++++**
	Total gbl.	++++	++++	++++	++++	**+++**
	Pseudogbl.	++++	++++	**++++**	++	±
	Eugbl.	++	+	+		
Colon.....................	Serum	++++	++++	++++	++++	**+++**
	Total gbl.	++++	++++	++++	**++++**	+
	Pseudogbl.	++++	++++	++++	**++++**	±
	Eugbl.	**++++**	++	–		

TABLE VI.

Exhaustion Experiment. Polyagglutinative Goat Serum.

Exhaustion with Flexner Manila of Fractions (Table V) on III/7/06.

Fraction.	Organism.	20	50	100	200	500	1000
Total gbl	Flexner Manila	**++++**	—	—	—	—	—
	Shiga	++++	++++	++++	++++	++++	**+++**
	Pfeiffer	++++	++++	++++	++++	++++	**+++**
	Cholera	++++	++++	++++	++++	++++	**++++**
Pseudogbl	Flexner Manila	—	—	—	—	—	—
	Shiga	++++	++++	++++	**++++**	++	
	Pfeiffer	++++	++++	++++	**++++**	+	
	Cholera	++++	++++	++++	++++	**+++**	
Eugbl	Flexner Manila	—	—	—	—	—	
	Shiga	++++	**+++**	+	—	—	
	Pfeiffer	++++	**+++**	—	—	—	
	Cholera	+	+	—	—	—	

Pick's corresponding experiment may be summed up at follows: As antityphoid goat serum agglutinating at 1 :2600 was precipitated in a series of progressively increasing concentrations of ammonium sulphate. The initial precipitation was at 2.6 saturation with a dilution of 1 :5, agglutination being evident at 1 :500 of the unfiltered precipitated mixture. At 3.4 saturation over half and at 3.6 saturation all the agglutinin was precipitated. Again, 20 c.c. of the same serum were precipitated with 10 c.c. of saturated ammonium sulphate solution; the euglobulin then agglutinated at 1 :2400 (in terms of original serum) the pseudoglobulin at 1 :20. After two reprecipitations the eu-fraction still reacted at 1 :2400.

Table VII.

Fractioning of Polyagglutinative Goat Serum.

Bleeding of III/18/06; fractioned III/20/06.

Organism.	Fraction.	100	200	500	1000
Flexner Manila	Total gbl.	++++	++++	**+++**	±
	Pseudogbl.	++++	**++++**	++	—
Shiga	Total gbl.	++++	++++	o	—
	Pseudogbl.	++++	++++	**++++**	±
Pfeiffer	Total gbl.	++++	**+++**	±	—
	Pseudogbl.	++++	**++++**	—	—
Cholera	Total gbl.	++++	**++++**	—	—
	Pseudogbl.	++++	**++++**	+	—
Colon	Total gbl.	++++	**+++**	—	—
	Pseudogbl.	++++	**+++**	—	—

Polyagglutinative Horse Serum.

(*a*) *Antidysentery Horse Serum.* Horse 284; immunized against the Shiga, Flexner Manila and the Mount Desert strains. Bleeding of X/3/06; fractioned X/4/06.

Here (Table VIII.), the agglutinins are split between the fractions, the larger part of each occurring in the pseudoglobulin.

(*b*) *Anticholera and Antityphoid Horse Serum.* Horse 254; combined immunization against the original Pfeiffer and cholera. Serum from bleeding on III/27/06; fractioned IV/1/06 and refractioned V/10/06. The results are given in Table IX.

With the cholera-typhoid serum, the agglutination values of which for each organism were in the neighborhood of a dilution of 1:1000, the bulk of the agglutinins was found in the pseudoglobulin (Table IX.); the greater portion was also in the high fraction when the serum was precipitated at 3.6 and again at 3.8 saturation. There is no evidence presented here that the precipitation limits of the cholera agglutinin are in any way different from that of the typhoid. In the second fractionation (V/10/06) it is seen that both the Pfeiffer and the cholera are increased in the eu-fraction as contrasted with the result of the first precipitation at 3.4 saturation.

TABLE VIII.

Fractioning of Antidystentery Horse Serum.

Organism.	Fraction.	50	100	200	500	1000	2000
Flexner Manila.....	Serum	++++	++++	++++	++++	**+++**	—
	Total gbl.	++++	++++	++++	**+++**	—	—
	Pseudogbl.	++++	++++	++++	**+++**	—	—
	Eugbl.	++++	++++	**+++**	±	—	—
Shiga	Serum	++++	++++	++++	++++	**+++**	—
	Total gbl	++++	++++	++++	**++++**	±	—
	Pseudogbl.	++++	++++	**++++**	—	—	—
	Eugbl.	++++	++++	**+++**	—	—	—
Colon...............	Serum	++++	++++	++++	++++	++	[illegible]
	Total gbl.	++++	++++	**++++**	—	—	—
	Pseudogbl.	++++	++++	**++++**	±	—	—
	Eugbl.	++++	**+++**	—	±	—	—

The fractionation of the anticholera and antityphoid horse serum is of especial interest because an experiment of this nature is the most striking of E. P. Pick's observations. Pick had found that the typhoid agglutinin was precipitated with the pseudoglobulin fraction in horse serum; the cholera agglutinin, on the contrary, came down with the euglobulin. Pick, therefore, mixed equal volumes of a typhoid and a cholera serum, and progressively precipitated 2 c.c. amounts of the mixed sera at 2.8, 3.0, 3.2, etc., saturation (with a final volume of 10 c.c. in each case). A well marked separation of the cholera agglutinin into the euglobulin and the typhoid agglutinin into the high fraction resulted. As given in Pick's tables, the observed agglutination values are twice too much, since each serum was diluted a half by the mixing. A direct separation at 3.4 saturation of the agglutinin in a goat cholera serum (agglutinating at a dilution of 1:1000) from the typhoid agglutinin in horse serum (1:20.000) was made. The euglobulin was precipitated by adding a half volume of saturated ammonium sulphate solution directly to the mixed sera. The high agglutination values are again given as *direct* observations, though probably calculated for the original undiluted sera. The figures for the reprecipitated euglobulin are for the typhoid, 1:3000, and for the cholera 1:1600 (an increase over the value of the original goat serum); the pseudoglobulin (3.3 saturation filtrate), agglutinated the typhoid at 1:16,000, the cholera at 1:20.

TABLE IX.

Fractioning of Anticholera-Antityphoid Horse Serum.

1. Precipitation at 3.4 saturation.

Organism.	Fraction.	50	100	200	500	1000
Pfeiffer	Total gbl.	++++	++++	++++	++++	**+++**
	Pseudogbl.	++++	++++	++++	**+++**	±
	Eugbl.	+	+	—	—	—
Cholera	Total gbl.	++++	++++	++++	++++	**++++**
	Pseudogbl.	++++	++++	+++	**+++**	+
	Eugbl.	+	+	—	—	—

2. Precipitation at 3.6 saturation.

Organism.	Fraction.	50	100	200	500	1000
Pfeiffer	Total gbl.	++++	++++	++++	**++++**	++
	Pseudogbl.	++++	++++	++++	**+++**	—
Cholera	Total gbl.	++++	++++	++++	++++	**++++**
	Pseudogbl.	++++	++++	++++	++++	**++++**

3. Precipitation at 3.8 saturation.

Organism.	Fraction.	50	100	200	500	1000
Pfeiffer	Total gbl.	++++	++++	++++	**++++**	±
	Pseudogbl.	++++	++++	+++	**+++**	±
Cholera	Total gbl.	++++	++++	++++	++++	**++++**
	Pseudogbl.	++++	++++	++++	**+++**	±

4. The same serum was again fractioned on V/10/06.

Organism.	Fraction.	50	100	200	500	1000	2000
Pfeiffer	Total gbl.	++++	++++	++++	++++		
	Pseudogbl.	++++	++++	++++	✚✚✚		
	Eugbl.	++++	++++	✚✚✚✚	—		
Pfeiffer*	Total gbl.	++++	++++	++++	++++	✚✚✚	++
	Pseudogbl.	++++	++++	++++	✚✚✚	±	-
	Eugbl.	++++	✚✚✚✚	—	—	—	—
Cholera............	Total gbl.	++++	++++	++++	++++	✚✚✚	
	Pseudogbl.	++++	++++	++++	✚✚✚	++	
	Eugbl.	++++	++++	✚✚✚	±	-	

* A second fractionation.

TABLE X.

Fractioning of Anticholera-Antityphoid Horse Serum.

Horse 254; bleedings of II/27/06 and V/29/06; fractioned X/8/06.

Fractions tested with the Mt. Sinai culture of typhoid.

Centrifuged.	Fraction.	100	200	500	1000	2000
	II/27/06					
2 hrs. after precipitation......	Total gbl.	++++	++++	++++	✚✚✚	-
	Pseudogbl.	++++	+++	✚✚✚	±	
	Eugbl.	++++	✚✚✚	—	±	
After 12 hrs.................	Total gbl.	++++	++++	++++	++++	✚✚✚
	Pseudogbl.	++++	+++	✚✚✚	±	
	Eugbl.	++++	✚✚✚	+	±	
	V/29/06					
	Serum	++++	++++	++++	✚✚✚✚	—
After 2 hrs..................	Total gbl.	++++	++++	++++	✚✚✚	—
	Pseudogbl.	++++	++++	✚✚✚✚	++	±
	Eugbl.	++++	++++	✚✚✚✚	++	±
After 12 hrs........	Total gbl,	++++	++++	++++	✚✚✚✚	+
	Pseudogbl.	++++	+++	✚✚✚	+	
	Eugbl.	✚✚✚✚	++	±		

In the following observations it is shown that a relatively large proportion of the typhoid agglutinin may occur in the euglobulin fraction.[1]

Six c.c. of each serum were diluted with 13.8 c.c. of water and precipitated by the gradual addition of 10.2 c.c. of saturated ammonium sulphate solution. Uniform samples (15 c.c.) of each were centrifuged after two hours' standing. The euglobulin precipitates were washed by thoroughly suspending the proteid in 15 c.c. of 3.4 saturation ammonium sulphate solution and again centrifuging; the precipitates were washed three times in this fashion. Exactly similar precipitations were made on the two sera, but the precipitated mixtures were centrifuged after twelve instead of two hours' standing. The results are given in Table X.

A high typhoid agglutinin content in the euglobulin was also obtained on fractioning the last bleeding of the cholera-typhoid horse. The results are shown in Table XI.

Table XI.

Fractioning of anticholera-antityphoid Horse Serum.

Horse 254; bled X/3/06; fractioned X/4/06; tested only with typhoid.*

Organism.	Fraction.	100	200	500	1000	2000
Mt. Sinai Typhoid..........	Total gbl.	++++	++++	++++	✚✚✚✚	++
	Pseudogbl.	++++	++++	++++	✚✚✚	–
	Eugbl.	++++	++++	✚✚✚	–	–
St. Typhoid....	Total gbl.	++++	++++	✚✚✚✚	+	±
	Pseudogbl.	++++	++++	✚✚✚✚	++	–
	Eugbl.	++++	++++	✚✚✚	–	–

* The Pfeiffer strain agglutinated spontaneously. The Mount Sinai strain has always shown the same agglutinations as the Pfeiffer in numerous other observations.

Fractionation of Diphtheria Antitoxic Goat and Horse Serum.

Fresh goat serum and serum from horse 307, VII/5/06, were fractioned as follows: 3 c.c. of serum diluted with 6.9 c.c. of water were precipitated with 5.1 c.c. of saturated ammonium sulphate solution, and the precipitate from a 10 c.c. sample of each obtained by centrifuging. The precipitates were suspended in 3 c.c. of 3.4 saturated ammonium sulphate solution and again centrifuged; the washing was twice repeated and the wash solutions united and made up to 10 c.c. The precipitates were suspended as usual in 3.4 saturated ammonium sulphate and made up to 10 c.c. The results calculated per c.c. of the original undiluted serum follow:

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[1] On resuming this problem in the fall of the year, it was found that our cholera culture was spontaneously agglutinating; it could not therefore be employed in testing the agglutination values of the fractions.

Fraction.	Goat.	Horse.
Serum	90 units	250 units
Total globulin	90 "	250 "
Pseudoglobulin	35 "	200 "
Euglobulin	5 "	0* "
Wash solution	50 "	25 "

* Tested for 5 units against 100 m. l. d.; the guinea pig died in 12 hours, autopsy showing a typica diphtheria toxin picture.

The same results were obtained in two similar experiments. The two sera certainly show a different behavior towards ammonium sulphate precipitation. A relatively large proportion of the goat antitoxin is precipitated with the euglobulin. The facility with which the antitoxin can be washed out almost completely (in a total united volume of wash solution less than the original volume of the precipitated mixture) shows that the antitoxin is not invariably linked to the euglobulin.

Pick's experiment is given briefly for comparison with our results:

Twenty c.c. of antitoxic goat serum[1] (neutral reaction) were precipitated with 10 c.c. of saturated ammonium sulphate solution. After two hours, the precipitate was pressed out and dissolved in 30 c.c. of water; it was reprecipitated at 3.3 saturation and dissolved in 20 c.c. of water (euglobulin fraction). The filtrates were united and made up to half saturation, the precipitate dissolved in the original volume (20 c.c.) and refractioned between 3.3 and 5.0. The euglobulin then obtained was united with the above euglobulin solution and the mixture precipitated at 3.3 saturation. The serum contained about 10 units per c.c. The fractions tested as follows:

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[1] Pick states (p. 361) "Zu dem nun folgenden Trennungsversuche mit Diphtherie immun Ziegenserum stand mir nur ein Ziegenserum zur Verfügung, von dem 0.1 c.cm. eben im Stande war, die 10-fache tödliche Giftmenge eines Toxins zu paralysieren das in der Dosis von 0.0098 c.cm. ein Meerschweinchen von etwa 260 g. in drei Tagen tötete." This would make the potency of the serum used only 1 unit per c.c. The control test made actually gives the strength as 10 antitoxin units per c.c.: 0.098 c.c. toxin (10 m.l.d.) were neutralized by 0.01 c.c. serum; therefore 100 m.l.d. toxin were neutralized by 0.1 c.c., or 1 c.c. serum neutralized 10 × 100 m.l.d. toxin. Ledingham has passed this over: "The goat serum with which Pick worked had a very low antitoxic value inasmuch as 0.1 c.c. was required to neutralize 10 lethal doses of a toxin whose m.l.d. was only about 0.01 c.c." Pick, himself, speaks of the antitoxic value of this serum incidentally "Man erkennt trotz der geringen Wertigkeit des Serums......"

Pseudoglobulin:

0.05 c.c. +10 m.l.d. toxin (testing for 2 units). The guinea pig died on the second day.

Euglobulin:

0.01 c.c. + 10 m.l.d. toxin (testing for 10 units). Died on the third day.

0.017 c.c. + 10 m.l.d. toxin (testing for 6 units). Induration and loss of weight, but survived.

Ledingham states in his conclusions that in the horse serum the relationship of the diphtheria antitoxin to the pseudoglobulin fraction "holds good only when the antitoxin content of the serum is steadily rising." Horse 307 of this department had been subjected to immunization for over five months; it attained a maximum of over 300 units per c.c. in three months and had declined to 250 units two months later when the blood of the serum used in our fractionation experiments was drawn.[1] Apparently Ledingham's conclusion (from observations on a single horse) is not of general application.

Tabulating Pick's results by a somewhat different arrangement than the one presented in his paper (p. 384), it is seen from the following—

Animal.	Diphtheria Antitoxin.	Tetanus Antitoxin.	Cholera Lysin (Pfeiffer).	Typhoid Agglutinin.	Cholera Agglutinin.
Goat	eugbl.	eugbl.	eugbl.	eugbl.	eugbl.
Rabbit				eugbl.	
Guinea pig				eugbl.	
Horse	pseudogbl.	pseudogbl.		pseudogbl.	eugbl.

—that there is no evidence of any differences in the precipitation limits of the antibodies in goat, rabbit and guinea pig sera. We should hardly expect *a priori*, then, that a separation of any other antibodies by fractionation of goat and rabbit serum would be possible, and we have not found otherwise. Yet the probability of such a separation for horse serum was suggested by Pick's experiments. With this serum, even, the distribution of the antibodies as determined by Pick, has been similarly homogeneous with the exception of the cholera agglutinin. Pick, accordingly, gives one example, and one only, of an antibody differing from other antibodies in the serum of the same species by its precipitation characters toward ammonium sulphate. This observation of Pick's we have been unable to verify when polyagglutinative horse sera have been used. It is probable, however, that the serum globulins of different animals or even of various individuals of the same species may show a different and inconstant behavior quantitatively toward fractional ammonium sulphate precipitation. We have not found that any of the antibodies in goat, rabbit and horse serum were invariably associated with the euglobulin. Our results with goat diphtheria antitoxin have been confirmed by Ledingham. At the same time we have presented repeated observations with several strains of typhoid showing that a large proportion (almost half in some instances) of the typhoid agglutinin of horse serum may be found in the thoroughly washed euglobulin fraction of both old and fresh serum.

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[1] The horse subsequently was killed as no longer of service for antitoxin production.

The results of our experiments have already been briefly summarized in the preceding portion of the paper.

It is to be hoped that any future work on the fractionation of the antibodies or of the proteids of the blood will not be undertaken without a thorough comprehension of the nature and limitations of the process. Salt fractionation is a valuable method for the purification and preparation of proteid products; the salt concentration precipitation limits, however, are not a reliable means for differently classifying proteids the precipitation characters of which are not widely separated.

THE PRODUCTION OF AGGLUTININS IN THE ANIMAL BODY BY THE INOCULATION OF SUBSTANCES OTHER THAN PRODUCTS OF BACTERIAL ORIGIN.*

By Katharine R. Collins, M. D.

There is to-day among observers working along the lines of immunity a tendency to no longer consider a given anti-body and the factors producing it as a compact inseparable entity following the laws of protoplasmic continuity, but as a complex body that may be split up into component parts, which parts may exhibit individuality of structure and independence of action. This view of the subject has been given an impetus by the work of Vaughan and later Obermeyer and Pick. Vaughan derived split products from bacterial proteids and egg-albumen, representing a poisonous and non-poisonous portion. Obermeyer and Pick found by iodizing protein they so changed it that when the iodized portion was inoculated into animals only non-specific precipitins were formed. This led them to believe that specificity in this case was due to the aromatic radical which was changed by iodization.

The following work was begun in 1905 with hope of determining through the nature of substances having the power to produce agglutinins in the animal a more intimate knowledge of the anti-bodies and their elaboration in the animal economy. This in a measure was realized. As the work progressed the fact developed that, as far as tested, certain molecules or radicals containing such molecules, are always present in the substances, which induce an increase of agglutinin production; this fact strongly suggests the possibility that these molecules are responsible, for one feature at least, of this phenomenon. The observations arrange themselves under the following heads: (1) organized ferments, (2) unorganized ferments, (3) metabolic products, (4) putrefactive products, (5) inorganic substances.

Organized Ferments—Ballner and Sagasser, in 1904, succeeded in producing, artificially, agglutinins for Bacillus typhosus by inoculating animals with red yeast cells (Rosa Hefe), but the yeast cells themselves were not agglutinated by the serum.

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* Read before the American Association of Pathologists and Bacteriologists, April 17, 1908.

I have not been able to identify the Rosa Hefe used by Ballner and Sagasser. The red yeasts mentioned in most works on fermentation is, strictly speaking, a torula and not a true yeast. Several rabbits were inoculated with the cells of the so-called red yeast obtained from the air. This did not give rise to an increase in the production of agglutinins, though later these same rabbits responded to inoculation of brewer's yeast cells. Acting upon this suggestion of Ballner, I inoculated rabbits with living cultures of brewer's yeast. After four or five inoculations the sera of these rabbits were tested with the following organisms:

1. B. dysenteriae (3 strains), Shiga, Flexner Manila, Park Mt. Desert.
2. B. typhosus (2 strains), Pfeiffer, Mt. Sinai.
3. B. Coli (2 strains) (Laboratory, Colon X.).
4. B. paratyphosus.
5. B. pyocyaneus.
6. B. proteus vulgaris.
7. S. cholerae.
8. Pneumococcus, one strain.
9. Streptococcus, one strain.
10. B. mallei.

The only organisms that reacted with the various immune sera were Flexner Manila—B. typhosus, Pfeiffer strain and Colon X. Several strains of brewer's yeast, differing in their action upon beerwort, were tested without presenting any appreciable variation in results. The brewer's yeast was obtained in pure cultures from the laboratory of the Brewer's Academy, New York City, and were cultivated on 10 per cent. beerwort agar. Living cells were used. Beerwort alone inoculated into rabbits was without effect.

The result of inoculations with yeast cells may be shown in the following table:

TABLE I.

	Normal Serum.			After 8 Inoculations.					
	10	20	50	10	20	50	100	200	500
Shiga	—	—		—	—				
Flexner Manila	+	+	—	++	++	++	++		
Park Mt. Desert	—	—		—	—	—			
Pfeiffer	+	—		++	++	++	++		
Colon ×	+	—		—	—				

	After 13 Inoculations.						After 18 Inoculations.						
	10	20	50	100	200	300	10	20	50	100	200	500	1000
Shiga	—						—						
Flexner Manila	++	++	++	++	++	+	++	++	++	++	++	++	++
Park Mt. Desert		– – –	—	—	—		—	—					
Pfeiffer	+	+					—						
Colon ×	—						—						

Several rabbits inoculated with yeast cells also gave the same results as the goat. The serum of these rabbits, when tested, showed a steady increase after inoculation, for the Flexner Manila strain of B. dysenteriae, an increase for B. typhosus and sometimes Colon X. strain B. coli with the subsequent disappearance of the two latter. This disappearance of the agglutinins for B. typhosus and Colon X. would seem to indicate one of two things. First, the normal cells of the rabit possess a greater potentiality for the manufacture of Flexner Manila agglutinins, on account of the cell being subjected to a stronger and more specific stimulation in this direction and therefore when influenced by non specific substances the cell forms agglutinins for this organism in preference to others because of this stronger initial stimulation. As the inoculations proceed the increment of production for the Flexner Manila strain becomes more marked and finally the entire energies of the cell seem to be occupied in forming these agglutinins to the exclusion of the others.

On the other hand the possibility suggests itself that the stimulating agent may not be so entirely non-specific as generally considered. This phase would then agree with that seen when definite specific organized agents such as bacteria or their products are used, stimulation of common agglutinins being induced. Common agglutinins may continue throughout to be formed to an equal degree along with the specific, or may disappear almost entirely after long immunization. This disappearance or persistence seems to depend somewhat upon the relation of the heterologous organisms to the homologous. The more nearly the species are related the more persistent the common agglutinins.

The serum of a rabbit immunized with yeast cells was subjected to the exhaustion test with B. typhosus, the Flexner Manila organism and living brewer's yeast cells—and cells of the torula. The agglutinins for Flexner Manila were completely exhausted by this organism, B. typhosus, and the yeast cells absorbed all but 10 per cent. of the Flexner Manila agglutinins. This amount not absorbed approximately represents the amount of normal agglutinins for Flexner Manila present in the serum, which, on account of its more specific nature, would resist the action of purely non-specific agents.

Unorganized Ferments—The possibility that other enzymes might bring about this increase in the animal of pre-existing agglutinins suggested the use of the unorganized ferments, diastase, pancreatin and invertin. The results obtained by the inoculation of these substances coincide with those obtained by the inoculation of yeast cells.

The agglutinins were in each case increased from 1:50 to 1:500 after five or six, weekly, inoculations. The enzymes, however, failed to absorb the agglutinins thus raised from the homologous serum.

Products of Metabolism—Nuclein as a component part of the yeast cell was first used. Vaughan obtained a certain amount of protection against the pneumococcus in guinea pigs by the inoculation of nuclein previous to the pneumococcus infection. The nuclein was used in the form of a nucleoproteid from the pancreas, for which I am indebted to Dr. Levene, of the Rockefeller Institute for Medical Research. The other products used of the metabolic group were lecithin and proteoses from egg. The same increase of agglutinins for Flexner Manila type of B. dysenteriae followed the inoculation of these substances as with yeast and enzymes. There was initial rise for Flexner Manila type, B. typhosus and B. coli, with the subsequent dropping out of the two latter and a continued rise of the former as the inoculations were continued.

Products of Putrefaction—The products of putrefaction tested were indol, skatol, ethyl mercaptan and phenol. I am indebted to Dr. C. A. Herter for the indol, skatol and mercaptan, and also for some valuable suggestions as to their use.

Ethyl mercaptan in one per cent. solution produced an increase of agglutinins in two rabbits after the fifth inoculation and followed the same course as in the cases where the enzymes and metabolic products were used. A control rabbit on injection of ethyl alcohol did not show an increased production of its normal agglutinins. Indol and skatol were without effect, as might have been expected on account of the readiness with which they combined with the preformed sulphates and were excreated. Phenol, however, did enter into systemic relation with the organism and toxic effects were demonstrated in the animals inoculated, but a rise of agglutinins did not occur after a number of inoculations, the animals finally dying from excessive abscess formation.

At this point two factors present themselves as possible influences in causing an increase in agglutinins. The first is the increased production and destruction of leucocytes and the second is the fact that the substances bringing about this increase of agglutinins with the exception of the enzymes, concerning whose structures little is known, all possess a formula containing phosphorus or sulphur molecules, while those failing to effect an increase do not possess either elements.

First the effect of the inoculations of these substances upon the leucocytes may be considered. Dieudonne claims that animals possessing agglutinins for a certain organism may have the amount of agglutinins increased by the inoculation of exciters of leucocytes, such as aleuronat and hetol. Aleuronat in our hands brought about an increase of the initial agglutinins similar to that caused by the substances used in the preceding experiments. Hetol was not used.

To test the effect of our inoculations upon leucocytes, blood counts were made upon normal rabbits, rabbits inoculated with substances increasing the agglutinins, and substances which did not effect the agglutinins. The blood counts exhibit the same irregularity in the normal rabbit and in the inoculated rabbits irrespective of the occurance or non-occurrance of an increase in agglutinins. This irregularity is in accordance with the statement of Brinckerhoff that the number of leucocytes per millimeter in the peripleral blood of the rabbit is constantly changing. It would appear from this irregularity that leucocytes could not account for the increased production. The action of aleuronat should, therefore, be ascribed to its behavior as a proteid rather than as an excitor of leucocytosis.

Inorganic Salts—To follow more in detail the suggestion developed by the fact that phosphorus and sulphur were the elements possessed in common by the augmentors of agglutinins, several soluble inorganic salts of sulphur and phosphorus were tested, and their action was controlled as far as practical by salts containing the same base but not the same radical.

Several rabbits were inoculated with sodium phosphate, sodium sulphate, calcium and potassium phosphate. Control rabbits were inoculated with sodium chloride, calcium chloride, and potassium chlorate. A young goat was inoculated with sodium sulphate and a control goat with sodium chloride. The sera of the rabbits and the goat receiving the sulphur and phosphorus compounds showed the characteristic increase of agglutinins for the Flexner Manila strain of B. dysenteriae. The agglutinins for B. typhosus and B. coli were only slightly stimulated in a few instances. The agglutinins of the control animals inoculated with the salts that did not contain the sulphur or phosphorus molecules were uneffected. The doses varied according to the toxicity of the salts used and the concentrations ranged from one-tenth normal to twenty-five per cent. solutions. The concentration of the solution had no apparent effect upon the results. It is interesting to note that Vaughan found the phosphorus in the non-toxic portion of the split products to be the part which gives immunity.

A question arises as to the character of the action of these substances. Do they merely stimulate to greater activity a specific function of the cell already established, or do they possess something in common with the bacteria which admits of their initiating specific action in some degree? An attempt was made to answer this question in the following manner:

Two rabbits each having normal agglutinins for Flexner Manila type of B. dysenteriae up to 1:50, but none that were appreciable for Shiga or Park Mt. Desert types, were inoculated with the Shiga type and the other with the Park Mt. Desert organism. After three inoculations the sera of these rabbits agglutinated their homologous organism in dilutions of 1:100. The index for Flexner Manila type remained unchanged. The bacterial inoculations were then stopped and sodium sulphate substituted in one rabbit and diastase in the other. After four or five treatments the sera from both rabbits were tested. The agglutinins for the Flexner Manila organism were increased up to 1:500, while those for Shiga and Park Mt. Desert type remained unchanged and later disappeared.

These two experiments are not sufficient, however, to prove or disprove the assumption that the action is one of augmentation and not of initiation. I have mentioned the foregoing question because it suggests several points to be taken into consideration in answering it. First, the influence that has brought about the normal agglutinins in the rabbit for Flexner Manila has been acting practically during the adult life of the animal, while the influence of the Shiga or Park Mt. Desert organisms has only been exerted for a comparatively short period of time. Hence the function of the cell to form the Flexner Manila agglutinins would be more permanent than for the other two organisms. Now, when the rabbits are inoculated with strong specific substances as Shiga or Park Mt. Desert, the cell responds accordingly as long as this stimulus is kept up. But upon removal of this influence and the substitution of a new, presumably, specific stimulus, then the cell by preference responds in the direction of the more accustomed function of forming agglutinins for Flexner Manila. Another point to be considered is the fact that after the withdrawal of the specific influences of Shiga and Park Mt. Desert the cause producing the normal agglutinins for Flexner Manila in the rabbit continues to act, thus adding its influence to the stimulus of the non-specific sodium sulphate inoculations and so determining the direction of the activity of the cells.

At this stage of the present investigation biological rather than physical laws seem to offer the most probable explanations of the production or augmentation of agglutinins which has been described; perhaps stimulation of certain cell activities occur because some necessary element which enters into the cell or acts as a ferment adjuvant is provided.

The work of several authors on ferments is suggestive by analogy. Bertrand found that manganese was present in laccase and activity of laccase was proportional to the amount of this salt present.

Calcium salts are found to be essential to enzymes which cause clotting and Magnus has given the name of co-ferments to those substances, but this term has been rejected by Hardin and Young. Hardin and Young found that boiled yeast which was capable of initiating fermentation alone when added to unboiled yeast increased its action to a considerable extent. They found this increase due to the presence of soluble phosphates. Arsenates brought about the same results as the phosphates.

Bibliography.

Ballner and Sagasser, Arch. für Hyg., 1904, li., 125.

Brinckerhoff, Jour. of Med. Res., 1904, v., 173.

Dieudonne, Med. Klin., No. xxii., 1906, 575.

Hardin and Young, Proc. Roy. Soc., Lond., Vols. 77 and 78, 1906.

Obermeyer and Pick, Wiener Klin. Woch., 1906, xix., 327.

Vaughan, Cellular Toxins, p. 100; Jour. of Infectious Diseases, 1907, iv., 476.

IS THE PRESENT METHOD OF STANDARDIZING ANTIDIPHTHERIC SERUM ACCORDING TO ANTITOXIN UNITS THERAPEUTICALLY ACCURATE?

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Cruveilhier[1] quotes Roux, Marfan, Martin and Momont, as finding that the dose of antidiphtheric serum most efficacious therapeutically is not always the one which contains the greatest number of antitoxic units. These opinions are contrary to those formed by the physicians observing the cases treated in the hospitals in New York City. The experiences of these authors, Cruveilhier says, seems to indicate that the serum contains, besides the antitoxin, other important preventive and curative substances. In the standardization of antidiphtheric serum as at present practiced, namely, according to antitoxic units, these protective substances are entirely ignored. Cruveilhier to test this point, carried out a series of experiments with guinea pigs, infected with diphtheria bacilli. He compared sera of different antitoxic strength from several horses as to their value preventively and curatively. In his preventive experiments he injected subcutaneously a quantity of serum proportional to the weight of the animal; and, 24 hours later, inoculated subcutaneously a fatal dose of diphtheria culture. He reported the following results: In four out of seven experiments, with culture No. 261, the animals which received 1/250,000 of 1 c.c., per gram weight, of a 200-unit serum resisted; while those which received the same volume quantity of a 500-unit serum died. In only one instance did the 500-unit serum prove superior, per volume quantity, to the 200-unit serum. Twice the results obtained per volume quantity for both sera were the same. On comparing a 50-unit with one of 500 units, the 50-unit serum proved superior to the 500-unit one, per volume quantity.

With two other cultures, designated "c.c." and "x," he compared a 200-unit serum with a 500-unit one, and obtained the same results.

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[1] *Ann. de l'Inst. Past.*, 1904, 18, p. 249.

Again, using culture No. 261, he compared a 300-unit serum with one of 500 units, from different bleedings of the same horse, and found them equal per volume in protection.

In his curative experiments, using the same cultures as in the preventive, he injected guinea-pigs subcutaneously with that amount of diphtheria culture which killed the control animal in from 36–48 hours. The animals were divided into two lots. At intervals of two hours, from the second to the sixteenth hour after inoculation, each animal of one lot received subcutaneously 0.1 c.c. of the serum of the lesser unit content, while each animal of the second lot received 0.1 c.c. of the serum of a greater unit content. In his curative experiments the sera of the lesser unit content were more efficacious than those of the greater.

These results apparently showed that it was the quantity of serum, rather than the number of antitoxic units, which was of therapeutic value.

Cruveilhier drew the following conclusions:

"Que l'effet curatif d'un sérum ne dépend pas exclusivement de sa teneur en unités antitoxiques.

"Que le titrage de l'antitoxine, tel qu'on le pratique habituellement, ne suffit pas à rendre un compte exact de l'efficacité d'un sérum. Que celle-ci est plus exactement appréciée parce que nous avons appelé la mesure du pouvoir thérapeutique."

In view of the large amount of work which has been done on the titration of diphtheria antitoxin, the results of Cruveilhier cannot but be considered extraordinary.

We have carefully investigated this subject and have obtained results diametrically opposed to those of Cruveilhier and the authors he cites.

In our experiments we compared both preventively and curatively, native sera and antitoxic globulin solutions (Gibson[1]) of the following unit content: A serum of 43 units compared with an antitoxic globulin solution of 1,700 units; a serum of 43 units with an antitoxic globulin solution of 1,450 units; a serum of 200 units with one of 1,000 units;

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[1] *Jour. Biol. Chem.*, 1906, p. 161.

a serum of 600 units with one of 1,300 units; a serum of 600 units with one of 335 units, also with one of 200 units. The last three sera were obtained from the same horse during the course of immunization.

The animals infected were active, healthy normal guinea-pigs weighing between 240 and 260 grams.

The diphtheria bacilli were from the three following strains: Culture No. 1, a moderate toxin producer; culture No. 2, a weak toxin producer. Both of these were freshly isolated from the throats of diphtheria patients at the Department of Health Hospital. Culture No. 8 (Park and Williams), is a strong toxin producer, which has been stock culture in this laboratory for 12 years.

In order to avoid misunderstanding, it should be stated that in the first seven preventive, and the first six curative experiments, the cultures in medium-sized tubes were grown on slant agar for 24 hours in the incubator. Of these, ⅛ of a culture was fatal to 250-gram guinea-pigs in 26 to 33 hours. Beginning with experiment 8 preventive, and with experiment 7 curative, the cultures were grown in *large* uniform test-tubes on slant agar. The fatal dose, thereupon, became 1/25 culture.

Preventive Experiments.

Following the technique of Cruveilhier, a serum[1] of 43 units per c.c. was compared with an antitoxic solution globulin[2] of 1,700 units per c.c.

Four guinea-pigs were injected subcutaneously with 1/150,000, 1/200,000, 1/250,000, 1/300,000 of a c.c. per gram weight, of the 43-unit serum, respectively. A parallel set of four guinea-pigs received the same volume per gram weight of the 1,700 unit antitoxic globulin solution; 24 hours later each animal in both series, as well as a control guinea-pig, received subcutaneously a fatal dose of culture No. 8. The control animal died in 24½ hours. The animals which received the 43-unit serum died in 25–28 hours, while those which received the antitoxic globulin solution remained normal. In the above experiment, the

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[1] Obtained from Horse 308. This animal had received increasinng amounts of toxin since March 6, 1906. The most potent antitoxic value was 250 units on May 3, 1906. Although receiving increasing amounts of toxin every day, the potency dropped steadily, and on July 7, 1906, seven days after the last injection of toxin (1,000 c.c. with a M. L. D. of 0.003), the antitoxic value was about 50 units. At the time of this experiment the serum tested 43 units per c.c.

[2] Prepared from 23 liters of citrated plasma (potency 700 units per c.c.) obtained from four bleedings of Horse 1.

1/150,000 c.c. per gram weight of the 43-unit serum contained 1/25 of a unit; while the same volume of antitoxic globulin solution contained 2¾ units.

In the second experiment, on account of the great difference in antitoxic unit content, the dilutions were made according to the number of units present.

Four guinea-pigs received 1/6,250, 1/12,500, 1/25,000 and 1/50,000 c.c. of the 43-unit serum, per gram weight, respectively. Two guinea-pigs received 1/425,000 and 1/850,000 c.c. per gram weight, respectively, of the 1,700-unit antitoxic globulin solution. Twenty-four hours later, all the animals, as well as a control, received a fatal dose of culture No. 8. Control animal died in 33-36 hours. The animal which received the 1/50,000 c.c. of the 43-unit serum showed marked induration and lost one-third of its weight. The two which received the 1/425,000 and 1/850,000 c.c. per gram weight of the antitoxic globulin solution remained normal.

In the third experiment made in conjunction with the second, a serum[1] of 200 units was compared with one[2] of 1,000 units.

Three guinea-pigs received 1/50,000, 1/100,000 and 1/200,000 c.c. of the 200-unit serum, per gram weight, respectively. Three others received 1/250,000, 1/500,000 and 1/1,000,000 c.c. of the 1,000-unit serum, per gram weight, respectively. Twenty-four hours later all the animals, as well as a control, received a fatal dose of culture No. 8. Control animal died in 33-36 hours. The animal which received 1/200,000 c.c. of the 200-unit serum and the one receiving 1/1,000,000 of the 1,000-unit serum showed marked induration and great loss of weight.

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[1] Obtained from Horse 307. This animal received increasing amounts of toxin since February 14, 1906. The most potent antitoxic value was 350 units on May 3, 1906. Two months later, on July 5, 1906, seven days after the last injection of toxin (850 c.c. with an M. L. D. of 0.003), the antitoxic value was 225 units. At the time of this experiment the serum tested 200 units per c.c.

[2] Obtained from Horse 305. This animal had received increasing amounts of toxin since February 14, 1906. The most potent antitoxic value was 1,250 units on April 17, 1906. On June 12, 1906, seven days after last injection of toxin (500 c.c. with an M. L. D. of 0.002) the antitoxic value was 1,100 units. At the time of this experiment the serum tested 1,000 units per c.c.

In the fourth experiment, four guinea-pigs received 1/25,000, 1/37,500, 1/50,000 and 1/75,000 c.c. of the 43-unit serum, per gram weight, respectively. Four others received 1/1,000,000, 1/1,500,000, 1/2,000,000, and 1/3,000,000 c.c. of the 1,700-unit antitoxic globulin solution, per gram weight, respectively. Twenty-four hours later all the animals as well as a control received a fatal dose of culture No. 8. Control died in 28 hours. The animal which received the 1/37,500 c.c. of the 43-unit serum was protected; the 1/50,000 c.c. failed to protect. The animal which received the 1/1,500,000 c.c. of the 1,700-unit antitoxic globulin solution was protected; the 1/2,000,000 c.c. failed to protect.

In the fifth experiment, four guinea-pigs received 1/100,000, 1/150,000, 1/200,000 and 1/250,000 c.c. of the 200-unit serum, per gram weight, respectively. Four others received 1/500,000, 1/750,000, 1/1,000,000 and 1/1,250,000 c.c. of the 1,000-unit serum, per gram weight, respectively. Twenty-four hours later all the animals, as well as a control, received a fatal dose of culture No. 8. Control animal died in 28 hours. The animal which received the 1/150,000 c.c. of the 200-unit serum was protected; the 1/200,000 c.c. failed to protect. The animal which received the 1/750,000 c.c. of the 1,000-unit serum was protected; the 1/1,000,000 c.c. failed to protect.

Thus far our results were not in accord with those of Cruveilhier. In our experiments the therapeutic value of the serum appeared to be measured by the antitoxic unit content, rather than, as his experiments indicated, by the quantity of serum.

TABLE I.

Preventive Experiments 1 *to* 5.

Fraction of c.c. per Gram Weight.	Serum of Horse 308 43 Units per c.c.	Fraction of c.c. per gram Weight.	Antitoxic Glob, Sol. 1,700 Units per c.c.
Experiment No. 1 with Culture 8.			
1/150,000	died	1/150,000	lived
1/200,000	died	1/200,000	lived
1/250,000	died	1/250,000	lived
1/300,000	died	1/300,000	lived
Control died in 24½ hours.			
Experiment No. 2 with Culture No 8.			
1/6,250	lived	1/425,000	lived
1/12,500	lived	1/850,000	lived
1/25,000	lived		
1/50,000	lived		
Control died in 33-36 hrs.			
Experiment No. 4 with Culture No. 8.			
1/25,000	lived	1/1,000,000	lived
1/37,500	lived	1/1,500,000	lived
1/50,000	died	1/2,000,000	died
1/75,000	died	1/3,000,000	died
Control died in 28 hrs.			

Fraction of c.c. per Gram Weight.	Serum of Horse 307 200 Units per c.c.	Fraction of c.c. per Gram. Weight.	Serum of Horse 305 1,000 Units per c.c.
Experiment No. 3 with Culture No. 8.			
1/50,000	lived	1/250,000	lived
1/100,000	lived	1/500,000	lived
1/200,000	lived	1/1,000,000	lived
Control died in 33-36 hrs.			
Experiment No. 5 with Culture No. 8			
1/100,000	lived	1/500,000	lived
1/150,000	lived	1/750,000	lived
1/200,000	died	1/1,000,000	died
1/250,000	died	1/1,250,000	died
Control died in 28 hrs.			

In experiment 6, the 43-unit serum was compared with an antitoxic globulin solution[1] of 1,450 units. Four guinea-pigs received ¾, ½, ¼ and ⅛ unit of the 43-unit serum, respectively. A parallel set of four guinea-pigs received the same fractions of a unit of the 1,450-unit antitoxic globulin solution. Twenty-four hours later all the animals as well as a control received a fatal dose of culture No. 1. Control animal died in 26 hours. The ¾ unit of both the serum and the antitoxic globulin solution protected, while all the animals which received the lower fractions died.

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[1] Prepared from 22 liters of citrated plasma (potency 600 units per c.c.) obtained from two bleedings each of Horses J and L.

Experiment 7 was identical with the preceding, except that culture No. 2 was used in place of culture No. 1. The results were: Control animal died in 25 hours. The animal which received the ½ unit of the 43-serum became greatly emaciated and barely survived. The ¾ unit of the same serum failed to protect. In all probability this difference was due to an idiosyncrasy of one of the animals. The ¾ unit of the 1,450-unit antitoxic globulin solution protected.

Experiments 6 and 7 were repeated in experiments 8 and 9. The antitoxic values injected were 1, ¾, ½, ¼, ⅛ and 1/16 unit. Using culture No. 1 for experiment No. 8, the results were: Control animal died in 26 hours. The ¾ unit of both the serum and antitoxin globulin solution protected.

In experiment 9, using culture No. 2, in place of culture No. 1, otherwise parallel with experiment 8, the results were: The control animal died in 23½ hours. The one unit of both the serum and the antitoxic globulin solution protected.

In experiment 10, using culture No. 1, a serum[1] of 600 units was compared with one[2] of 1,300 units. The same antitoxic values were administered as in experiments 8 and 9. The results were: Control animal died in 23 hours. The one unit of both sera protected.

In experiment 11, using culture No. 2, in place of culture No. 1, otherwise parallel with experiment 10, the results were: Control animal died in 33-36 hours. The ¼ unit of both sera barely protected.

In experiment 12, using culture No. 8, in place of No. 2, otherwise parallel with experiments 10 and 11, the results were: Control animal died in 28 hours. The ¾ unit of the 600-unit serum protected; the ½ unit of the 1,300-unit serum barely protected.

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[1] Obtained from Horse N. This animal had received increasing amounts of toxin since October 5, 1906. The most potent antitoxic value was 700 units on February 26, 1907, seven days after the last injection of toxin. Rebleeding one week later, the potency had dropped to 600 units. This was its value also at the time of our experiments, two weeks later.

[2] Obtained from Horse 306. This animal had received increasing amounts of toxin since February 14, 1906. The most potent antitoxic value was 1,450 units on May 18, 1906, six days after last injection of toxin (550 c.c. with an M. L. D. of 0.002). At the time of these experiments the serum tested 1,300 units per c.c.

In the two following experiments (13 and 14), comparisons were made of the three sera[1] of different unit content obtained from the same horse during the course of immunization.

In experiment 13, using culture No. 1, five guinea-pigs received 1, ¾, ½, ¼ and ⅛ unit of a 600-unit serum respectively, five others received the same antitoxic unit values of a 335-unit serum; five more received the same values of a 200-unit serum. Twenty-four hours later all the animals, as well as a control, received a fatal dose of culture No. 1. The results were: Control animal died in 28 hours. The ½ unit of each of the three sera protected.

In experiment 14, using culture No. 8 in place of culture No. 1, otherwise parallel with experiment No. 13, the results were: Control animal died in 30 hours. The ½ unit of each of the three sera protected.

To summarize: In experiments 1, 2, 4, 6, 7, 8 and 9, we compared a 43-unit native serum with a 1,700-unit and a 1,450-unit antitoxin globulin solution. The results, in each case, are seen to depend on the antitoxic unit content of the serum and not on its volume. The same is true of experiments 3 and 5, in which a 200-unit serum was compared with one of 1,000 units, and of experiments 10, 11 and 12, in which a fresh serum (two weeks old), of 600 units was compared with one (10 months old), of 1,300 units. Finally the same results are seen in experiments 13 and 14, in which we compared three bleedings from the same horse as follows: One bleeding originally contained 625 units per c.c. (this was the maximum value attained by the animal). The second contained 335 units per c.c., and the third contained 200 units per c.c.

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[1] Obtained from Horse 322. This animal had received increasing amounts of toxin since March 7, 1907. The most potent antitoxic value was 625 units, on April 30, 1907. Tested at the time of these experiments the serum contained 600 units per c.c. After the fourth bleeding, which was on July 7, 1907, the antitoxic value had dropped to 340 units; tested at the time of these experiments the serum contained 335 units per c.c. After three further bleedings, the antitoxic value dropped to 200 units on June 28, 1907. This was the potency also at the time of these experiments.

TABLE 2.

Preventive Experiments 6 *to* 14.

Amount Injected in Units.	Experiment No. 6 with Culture No. 1		Experiment No. 10 with Culture No. 1		Experiment No. 13 with Culture No. 1		
	Serum of Horse 308 43 Units per c.c.	Antitoxic Glob. Sol. 1,415 Units per c.c.	Serum of Horse N 600 Units per c.c.	Serum of Horse 306 1,300 Units per c.c.	Serum of Horse 322 600 Units per c.c.	Serum of Horse 322 335 Units per c.c.	Serum of Horse 322 200 Units per c.c.
1 unit			lived	lived	lived	lived	lived
¾ ··	lived	lived	died	died	lived	lived	lived
½ ··	died	died	died	died	lived	lived	lived
¼ ··	died	died	died	died	died	died	died
⅛ ··	died	died	died	died	died	died	died
1/16 ··			died	died			
	Control died in 26 hrs.		Control died in 23 hrs.		Control died in 28 hrs.		
	Experiment No. 7 with Culture No. 2		Experiment No. 11 with Culture No. 2		Experiment No. 14 with Culture No. 8		
1 unit			lived	lived	lived	lived	lived
¾ ··	died	lived	lived	lived	lived	lived	lived
½ ··	lived	died	lived	lived	lived	lived	lived
¼ ··	died	died	lived	lived	died	died	died
⅛ ··	died	died	died	died	died	died	died
1/16 ··			died	died			
	Control died in 25 hrs.		Control died in 33-36 hrs		Control died in 30 hrs.		
	Experiment No. 8 with Culture No. 1		Experiment No. 12 with Culture No. 8				
1 unit	lived	lived	lived	lived			
¾ ··	lived	lived	lived	lived			
½ ··	died	died	died	lived			
¼ ··	died	died	died	died			
⅛ ··	died	died	died	died			
1/16 ··	died	died	died	died			
	Control died in 26 hrs.		Control died in 28 hrs.				
	Experiment No. 9 with Culture No. 2						
1 unit	lived	lived					
¾ ··	died	died					
½ ··	died	died					
¼ ··	died	died					
⅛ ··	died	died					
1/16 ··	died	died					
	Control died in 23½ hrs.						

Curative Experiments.

Experiment 1—Following Cruveilhier's technique, we infected the animals with culture No. 8, and divided them into two lots. At intervals of two hours, from the second to the tenth hour, each animal of the first lot received subcutaneously 25 units of the 43-unit serum; and each animal of the second lot, 25 units of the 1,700-unit antitoxic globulin solution. The results of both lots were uniform. Control animal died in 25 hours. The animals which received the curative dose after 2 and 4 hours lived; while those receiving the curative dose after 6, 8 and 10 hours, died.

Experiment 2—The animals were infected with culture No. 1, and divided into two lots. At intervals of two hours, from the second to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 43-unit serum; and each animal of the second lot, 25 units of the 1,450-unit antitoxic globulin solution. The results of both lots were uniform. Control animal died in 26 hours. The animals which received the curative dose after 2, 4 and 6 hours lived; while those receiving the curative dose after 8 hours, died.

Experiment 3—The animals were infected with culture No. 2, in place of culture No. 1; otherwise parallel with experiment 2. The results of both lots were again uniform. The control animal died in 26 hours. The animals which received the curative does after 2 and 4 hours lived; after 6 and 8 hours, died.

Experiment 4—Repeating experiment 2, in duplicate, the result of both lots were again uniform. The control animal died in 21½ hours. The animals which received the curative dose after 2 and 4 hours lived; after 6 and 8 hours, died.

Experiment 5—Repeating experiment 3, in duplicate, the results were again uniform. The control animal died in 28 hours. The animals which received the curative dose after 2, 4 and 6 hours, lived; after 8 hours, died.

Experiment 6—The animals were injected with culture No. 1 and divided in two lots. At intervals of two hours, from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 600-unit serum. The second lot received 25 units of the

1,300-unit serum. The results of both lots were uniform. The control animal died in 27 hours. The animals which received the curative dose after 4 and 6 hours, lived; after 8 hours, died.

Experiment 7—The animals were infected with culture No. 2 in place of culture No. 1, otherwise parallel with experiment No. 6. The results of both lots were uniform. The control animal died in 28 hours. The animals which received the curative dose after 4 and 6 hours lived; after 8 hours, died.

TABLE 3.

Curative Experiments 1 to 11.

Hours Elapsed after Injection of Culture.	Experiment 1 with Culture 8.		Experiment 2 with Culture 1.		Experiment 6 with Culture 1.		Experiment 9 with Culture 1.		
	Serum of Horse 308 43 Units per c.c. (25 Units Injected).	Antitoxic Glob. Sol. 1,700 Units per c.c. (25 Units Injected).	Serum of Horse 308 43 Units per c.c. (25 Units Injected).	Antitoxic Glob. Sol. 1,450 Units per c.c. (25 Units Injected).	Serum of Horse N 600 Units per c.c. (25 Units Injected).	Serum of Horse 306 1,300 Units per c.c. (25 Units Injected).	Serum of Horse 322 600 Units per c.c. (25 Units Injected).	Serum of Horse 322 335 Units per c.c. (25 Units Injected).	Serum of Horse 322 200 Units per c.c. (25 Units Injected).
2 hours	lived	lived	lived	lived					
4 "	lived	lived	lived	lived	lived	lived	lived	lived	lived
6 "	died	died	lived	lived	lived	lived	died	died	died
8 "	died	died	died	died	died	died	died	died	died
10 "	died	died							
	Control died in 25 hrs.		Control died in 26 hrs.		Control died in 27 hrs.		Control died in 22 hrs.		
	Experiment 1 with Culture 8.		Experiment 3 with Culture 2.		Experiment 7 with Culture 2.		Experiment 10 with Culture 8.		
2 hours			lived	lived					
4 "			lived	lived	lived	lived	lived	lived	lived
6 "			died	died	lived	lived	died	died	died
8 "			died	died	died	died	died	died	died
			Control died in 26 hrs.		Control died in 28 hrs.		Control died in 24½ hrs.		

TABLE 3—(*Continued*).

Hours Elapsed after Injection of Culture.	Experiment 1 with Culture 8.		Experiment 4 with Culture 1 (in Duplicate).		Experiment 8 with Culture 8.		Experiment 11 with Culture 2.		
	Serum of Horse 308 43 Units per c.c. (25 Units Injected).	Antitoxic Glob. Sol. 1,700 Units per c.c. (25 Units Injected).	Serum of Horse 308 43 Units per c.c. (25 Units Injected).	Antitoxic Glob. Sol. 1,450 Units per c.c. (25 Units Injected).	Serum of Horse N 600 Units per c.c. (25 Units Injected).	Serum of Horse 306 1,300 Units per c.c. (25 Units Injected).	Serum of Horse 322 600 Units per c.c. (25 Units Injected).	Serum of Horse 322 335 Units per c.c. (25 Units Injected).	Serum of Horse 322 200 Units per c.c. (25 Units Injected).
2 hours			2 lived	2 lived					
4 "			2 lived	2 lived	lived	lived	lived	lived	lived
6 "			2 died	2 died	lived	lived	lived	lived	lived
8 "			2 died	2 died	died	died	died	died	died
			Control died in 21½ hrs.		Control died in 27 hrs.		Control died in 27 hrs.		
			Experiment 5 with Culture 2 (in Duplicate).						
2 hours			2 lived	2 lived					
4 "			2 lived	2 lived					
6 "			2 lived	2 lived					
8 "			2 died	2 died					
			Control died in 28 hrs.						

Experiment 8—The animals were infected with culture No. 8, in place of culture No. 2, otherwise parallel with experiments 6 and 7. The results of both lots were uniform. The control died in 27 hours. The animals which received the curative dose after 4 and 6 hours lived; after 8 hours, died.

Experiment 9—The animals were infected with culture No. 1 and divided into three lots. At intervals of two hours, from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 600-unit serum, the second lot, 25 units of the 335-unit serum, and the third lot received the same number of units of the 200-unit serum. These three sera were obtained from the same horse during the course of immunization. The results of the three lots were uniform. The control died in 22 hours. The animals which received the curative dose after 4 hours lived; after 6 and 8 hours, died.

Experiment 10—The animals were infected with culture No. 8 in place of culture No. 1, otherwise parallel with experiment 9. The results of the three lots were uniform. The control animal died in 24½ hours. Those which received the curative dose after 4 hours lived; after 6 and 8 hours, died.

Experiment 11—The animals were infected with culture No. 2, in place of culture No. 1; otherwise parallel with experiments 9 and 10. The results of the three lots were uniform. The control died in 27 hours. Those which received the curative dose after 4 and 6 hours lived; after 8 hours, died.

Conclusions.

In view of the results obtained in the comparison of the antitoxic content of sera, both preventively and curatively, it is obvious that in diphtheria in the guinea-pig, the therapeutic value of antidiphtheric sera depends on the number of antitoxic units present. There is no reason for believing that conditions in man are different.

According to our experiments antidiphtheric serum contains no protective substances, aside from the antitoxin, which play an important rôle therapeutically.

The present method of standardizing antidiphtheric serum accurately measures its therapeutic value.

THE RELATIVE THERAPEUTIC VALUE OF ANTITOXIC GLOBULIN SOLUTION AND THE WHOLE SERUM FROM WHICH IT WAS DERIVED.

DRS. EDNA STEINHARDT and EDWIN J. BANZHAF.

The process of concentrating diphtheria antitoxin, devised in this laboratory by Gibson,[1] has been adopted by a large number of American manufacturers, and has resulted in the extensive employment of the product in this country. Careful clinical trials have convinced us that this antitoxic globulin preparation possesses all the therapeutic properties of the native serum.

Cruveilhier[2] has recently stated that the customary method of standardizing antidiphtheric serum is not comprehensive enough, in that it leaves out of account therapeutic serum-constituents other than the antitoxin. While we believe that the work of Steinhardt and Banzhaf in the preceding paper[3] has effectually disposed of this objection, it seemed advisable to study the question in connection with the Gibson process of concentration, and with the fractional precipitation of the antiserum according to the method of Banzhaf and Gibson.[4]

Our general method of procedure was similar to that outlined in the preceding paper. In this instance, we mixed antitoxic citrated plasma from several horses and then tested this mixture before and after eliminating the non-antitoxic proteins. We also compared the different globulin fractions of the plasma.

In these experiments, the following mixtures of citrated plasma were used:

Mixture 1—Fifty liters of citrated plasma were obtained from five horses as follows:

1,000 c.c.	from horse	262,	part of one bleeding.
3,600 c.c.	“ “	305,	“ “ two bleedings.
5,400 c.c.	“ “	306,	“ “ three bleedings.
1,500 c.c.	“ “	310,	“ “ one bleeding.
38,500 c.c.	“ “	311,	full six bleedings.

The potency of this mixture, at the time of these experiments, was 700 units per c.c.

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[1] *Jour. Biol. Chem.*, 1, p. 161.
[2] *Ann. de l'Inst. Past.*, 1904, 18, p. 249.
[3] *Jour. Infect. Dis.*, 1908, 5, p. 203.
[4] *Jour. Biol. Chem.*, 3, p. 253.

Mixture 2—Forty liters of citrated plasma were obtained from three horses as follows:

8,000 c.c. from horse 288, full bleeding.
6.100 c.c. " " 305, full bleeding.
25,900 c.c. " " 299, full four bleedings.

The original potency of this mixture was 475 units per c.c. At the time of these experiments, however, the potency had dropped to 450 units per c.c.

(Mixture 1.) The 50 liters were refined and concentrated by Gibson's method, to 10 liters of antitoxic globulin solution containing 1,650 units per c.c.

Gibson's method briefly is as follows: The diluted citrated plasma is precipitated with an equal amount of saturated ammonium sulphate solution and the antitoxic proteins are separated by extracting the precipitate with saturated sodium chloride solution. The soluble antitoxic proteins are then reprecipitated from the saturated sodium chloride solution with acetic acid. This filtered precipitate is then partially dried between filter papers and dialyzed in parchment in running water.

(Mixture 2.) The 40 liters were divided into two lots. One lot of 20 liters was diluted with an equal amount of water and refined and concentrated by Gibson's method. The final product, designated 77A, amounted to 5.200 c.c., each c.c. containing 1,450 units per c.c. At the time of these experiments, however, the potency had dropped to 1,375 units per c.c.

The second lot of 20 liters was diluted with an equal amount of water and the antitoxic globulins obtained by three fractional precipitations at concentrations corresponding to 3.3 c.c., 3.3–3.8 c.c. and 3.8–5.0 c.c. of saturated ammonium sulphate in 10 c.c.[1]

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[1] There exists at the present time considerable confusion in comprehending the methods and basic principles of ammonium sulphate fractional precipitation of proteins. The nomenclature which we have employed and which designates the number of c.c. of saturated ammonium sulphate solution in 10 c.c. of the *precipitated* mixture has been used by some authorities; it avoids the confusion developed by the use of such terms as "per cent $(NH_4)_2SO_4$ solution," per cent saturation $(NH_4)_2SO_4$," "per cent of saturated $(NH_4)_2SO_4$ solution," and "per cent saturation $(NH_4)_2SO_4$ solution," and it seems the simplest and best *practical* expression of degrees of saturation yet suggested. We advise that this method be employed in future papers on fractional precipitation (see page 98).

The soluble antitoxic proteins in saturated sodium chloride solution were prepared in the usual way. The antitoxic globulin solution from the first fraction, designated 77B, amounted to 1,440 c.c., each c.c. containing 1,150 units per c.c. At the time of these experiments, however, the potency had dropped to 1,025 units per c.c. The second fraction, designated 77C, amounted to 1,400 c.c., each c.c. containing 1,350 units per c.c. At the time of these experiments, however, its potency had dropped to 1,175 units per c.c. The third fraction, designated 77D, amounted to 2,050 c.c., each c.c. containing 1,750 units per c.c. At the time of these experiments, however, the potency had dropped to 1,550 units per c.c.

The animals used in these experiments were active, healthy, normal guinea-pigs weighing between 250–260 grams.

The three diphtheria strains were the same as in the preceding paper.

PREVENTIVE EXPERIMENTS.

Mixture 1.

A uniform sample of the mixture of citrated plasma containing 700 units per c.c., was compared with the finished product of antitoxic globulin solution containing 1,650 units per c.c.

Experiment 1—Six guinea-pigs were inoculated subcutaneously with 1, ¾, ½, ¼, ⅛ and 1/16-unit of the 700-unit citrated plasma, respectively; a parallel set of six guinea-pigs received the same antitoxic unit values of the 1,650 antitoxic globulin solution. Twenty-four hours later all the animals, as well as a control guinea-pig, received a fatal dose of culture No. 1.[1] The control animal died in 27 hours. The ¾-unit of both the citrated plasma and the antitoxic globulin solution protected.

Experiment 2—In this, culture No. 2[1] was used in place of culture No. 1; otherwise it was parallel with the first experiment. The results were: The control animal died between 33 and 36 hours. The ¼-unit of both the citrated plasma and the antitoxic globulin solution protected.

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[1] Culture Nos. 1 and 2: No. 1, a moderate toxin producer; No. 2, a weak toxin producer. Both of these were freshly isolated from the throats of diphtheria patients at the Department of Health Hospital.

Experiment 3—In this, culture No. 8[1] was used in place of culture No. 2; otherwise it was parallel with the first and second experiments. The results were: The control animal died in 28 hours. The ½-unit of both the citrated plasma and the antitoxic globulin solution protected.

TABLE I.

Preventive Experiments.

Amount Injected in Units.	Experiment 1 with Culture No. 1.		Experiment 4 with Culture No. 1.				
	Sample of Plasma 700 Units per c.c.	Concentrated Antitoxic Glob. Sol. 1,650 Units per c.c.	Sample of Plasma 450 Units per c.c.	Concentrated Antitoxic Glob. Sol. 77A 1,375 Units pe6 c.c.	First Fract. Antitoxic Glob. Sol. 77B 1,025 Units per c.c.	Second Fract. Antitoxic Glob. Sol. 77C 1,175 Units per c.c.	Third Fract. Antitoxic Glob Sol. 77D 1,550 Units per c.c.
1 unit	lived	lived	lived	lived	lived	lived	lived
¾ “	lived	lived	lived	lived	lived	lived	lived
½ “	died	died	lived	lived	lived	lived	lived
¼ “	died	died	died	died	died	died	died
⅛ “	died	died	died	died	died	died	died
$\frac{1}{16}$ “	died	died	died	died	died	died	died
	Control died in 27 hrs.		Controls died in 28½ and 29 hrs.				
	Experiment 2 with Culture No. 2.		Experiment 5 with Culture No. 2.				
1 unit	lived	lived	lived	lived	lived	lived	lived
¾ “	lived	lived	lived	lived	lived	lived	lived
½ “	lived	lived	lived	lived	lived	lived	lived
¼ “	lived	lived	died	died	died	died	died
⅛ “	died	died	died	died	died	died	died
$\frac{1}{16}$ “	died	died	died	died	died	died	died
	Control died in 33-36 hrs.		Controls died in 28½ hrs.				
	Eoperiment 3 with Culture No. 8.		Experiment 6 with Culture No. 8.				
1 unit	lived	lived	lived	lived	lived	lived	lived
¾ “	lived	lived	lived	lived	lived	lived	lived
½ “	lived	lived	lived	lived	lived	lived	lived
¼ “	died	died	lived	lived	lived	lived	lived
⅛ “	died	died	died	died	died	died	died
$\frac{1}{16}$ “	died	died	died	died	died	died	died
	Control died in 28 hrs.		Controls died in 30-32 hrs.				

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[1] Park and Williams, Culture No. 8. This is a strong toxin producer and has been the stock in this laboratory for 12 years.

Mixture 2.

In Experiments 4, 5 and 6, we instituted the following comparisons:

(*a*) Between a sample of citrated plasma (Mixture 2) containing 450 units per c.c., and the finished product of antitoxic globulin solution containing 1,375 units per c.c.

(*b*) Between the same citrated plasma (Mixture 2), and the three fractions of the antitoxic globulins; of these Fraction 1 contained 1,025 units per c.c.; Fraction 2 contained 1,175 units per c.c.; and Fraction 3, 1,550 units per c.c.

Experiment 4—This required 30 guinea-pigs as follows: Six guinea-pigs received 1, ¾, ½, ¼, ⅛ and 1/16-unit of the 450-unit citrated plasma, respectively: six received the same antitoxic unit values of the 1,375-unit antitoxic globulin solution, 77A; six received the same antitoxic values of the 1,025-unit, first fraction, antitoxic globulin solution; six received the same antitoxic values of the 1,175-unit, second fraction, antitoxic globulin solution; six received the same values of the 1,550-unit, third fraction, antitoxic globulin solution. Twenty-four hours later, all the animals, as well as two control guinea-pigs, received a fatal dose of culture No. 1. The results of the five lots were uniform. The control animals died between 28½ and 29 hours. The ½-unit of the citrated plasma, the antitoxic globulin solution, and the fractions of the antitoxic globulins protected.

Experiment 5—In this, culture No. 2 was used in place of culture No. 1; otherwise it was parallel with experiment 4. The results of the five lots were uniform. The control animals died in 28½ hours. The ½-unit of the citrated plasma, the antitoxic globulin solution, and the fractions of the antitoxic globulins protected.

Experiment 6—In this, culture No. 8 was used in place of culture No. 2; otherwise it was parallel with experiments 4 and 5. The results of the five lots were uniform. The control animals died between 30 and 32 hours. The ¼-unit of the citrated plasma, the antitoxic globulin solution, and the fractions of the antitoxic globulins protected.

CURATIVE EXPERIMENTS.

Mixture 1.

The guinea-pigs were inoculated subcutaneously with a fatal dose of culture No. 1, and divided into two lots. At intervals of two hours from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 700-unit citrated plasma and each animal of the second lot received 25 units of the 1,650-unit antitoxic globulin solution. The results were: The control animal died in 27 hours. The animals receiving the curative dose after four and six hours, lived; while those receiving the curative dose after eight hours, died.

TABLE 2.

Curative Experiments.

No. of Hours after Injection of Culture	Experiment 1 with Culture No. 1		Experiment 4 with Culture No. 1				
	Sample of Plasma 700 Units per c.c. (25 Units Injected)	Concentrated Antitoxic Glob. Sol. 1,650 Units per c.c. (25 Units Injected)	Sample of Plasma 450 Units per c.c. (25 Units Injected)	Concentrated Antitoxic Glob. Sol. 77A 1,375 Units per c.c. (25 Units Injected)	First Fract. Antitoxic Glob. Sol. 77B 1,025 Units per c.c. (25 Units Injected)	Second Fract. Antitoxic Glob. Sol. 77C 1,175 Units per c.c. (25 Units Injected)	Third Fract. Antitoxic Glob. Sol. 77D 1,550 Units per c.c. (25 Units Injected)
4 hours	lived	lived	lived	lived	lived	lived	lived
6 "	lived	lived	lived	lived	lived	lived	lived
8 "	died	died	died	died	died	died	died
	Control died in 27 hrs.		Controls died in 28 hrs.				
	Experiment 2 with Culture No. 2		Experiment 5 with Culture No. 2				
4 hours	lived	lived	lived	lived	lived	lived	lived
6 "	lived	lived	lived	lived	lived	lived	lived
8 "	died	died	died	died	died	died	died
	Control died in 26½ hrs.		Controls died in 27-27½ hrs.				
	Experiment 3 with Culture No. 8		Experiment 6 with Culture No. 8 (in Duplicate)				
4 hours	lived	lived	2 lived	2 lived	2 lived	2 lived	2 lived
6 "	died	died	2 died	2 died	2 died	2 died	2 died
8 "	died	died	2 died	2 died	2 died	2 died	2 died
	Control died in 25½ hrs.		Controls died in 25 hrs.				

Experiment 2—In this, culture No. 2 was used in place of culture No. 1; otherwise it was parallel with the first experiment. The results were: The control animal died in 26½ hours. The animals receiving the curative dose after four and six hours, lived; while those receiving the curative dose after eight hours, died.

Experiment 3—In this, culture No. 8 used in place of culture No. 2; otherwise it was parallel with experiments 1 and 2. The results were: The control animal died in 25½ hours. The animals receiving the curative dose after four hours, lived; after six and eight hours, died.

Mixture 2.

Experiment 4—The guinea-pigs received subcutaneously a fatal dose of culture No. 1, and were divided into five lots. At intervals of two hours from the fourth to the eighth hour, each animal of the first lot received subcutaneously 25 units of the 450-unit citrated plasma; each animal of the second lot 25 units of the 1,375-unit antitoxic globulin solution, 77A; the third lot 25 units of the 1,025-unit, first fraction, antitoxic globulin solution; the fourth lot 25 units of the 1,175-unit, second fraction, antitoxic globulin solution; the fifth lot 25 units of the 1,550-unit, third fraction, antitoxic globulin solution. The results of the five lots were uniform. The control animals died in 28 hours. All the animals receiving the curative dose after four and six hours, lived; after eight hours, died.

Experiment 5—In this, culture No. 2 was used in place of culture No. 1; otherwise it was parallel with experiment 4. The results of the five lots were again uniform. The control animals died in 27 and 27½ hours. All the animals receiving the curative dose after four and six hours, lived; after eight hours, died.

Experiment 6—In this, which was in duplicate, culture No. 8 was used, in place of culture No. 2; otherwise it was parallel with experiments 4 and 5. The duplicate results of the five lots were again uniform. The control animals died in 25 hours. All the animals receiving the curative dose after four hours, lived; after six and eight hours, died.

In view of the results obtained in these experiments and in those of Steinhardt and Banzhaf in the preceding paper, it is obvious that the therapeutic value of the plasma is not appreciably impaired through the process of eliminating the albumins and other non-antitoxic proteins by the salting out methods employed, and the final dialyzation of the concentrated product.

A NOTE ON ANAPHYLAXIS.

By Drs. Edwin J. Banzhaf and L. W. Famulener.

Rosenau and Anderson in their first communication on hypersusceptibility reported their attempts to destroy or remove the toxic substances from horse serum. They treated the serum, which was used for the second injection, by various physical and chemical agents, such as heating the serum to 60 degrees C., filtering through porcelain, drying, freezing, precipitation and dialysis, and by the direct addition to the serum for various lengths of time of such chemicals as potassium permanganate, hydrogen peroxide, succinic acid and butyric acid, also various antiseptics, all without success. Continuing their investigations they reported, in their second communication, the influence of ferments, alkaloids, salts and such substances as ox bile, animal charcoal and yeast cells. These also gave negative results.

One of us took up the problem from another standpoint, *i. e.*, to treat the sensitized animal with drugs just prior to the second injection. In the preliminary experiments morphine sulphate was used with negative results. Following this, chloral hydrate was employed to produce hypnosis, and it was found that sensitized guinea pigs could be protected with this drug. After our experiments with chloral hydrate were well advanced, Besredka's communication came to our attention, in which he stated that ether and also calcium chloride exerted a protective action when adminstered to sensitized guinea-pigs before the second injection of the serum.

We found by injecting a solution of chloral hydrate which was just sufficient to produce hypnosis, that fully 75 per cent. of all serum sensitized guinea pigs were completely protected from the second injection of serum into the peritoneal cavity, while 90 per cent. of all the controls died. We believe that with improved technique in the dosage of chloral hydrate it is possible to protect 90 per cent. of all fully sensitized guinea-pigs. By "fully sensitized," we mean that three weeks or a month should elapse before the second injection of serum into guinea-pigs which have survived the routine testing of antitoxin. With guinea-pigs which have received horse serum alone (1/100 to 1/500 c.c.) at least seven or eight weeks should elapse before the second injection. By allowing the above interval of time to elapse, over 90 per cent. of our controls died within an hour, most of them within 20 minutes.

We have found that the dose of the chloral hydrate per gram weight of the animal was more or less variable, no fixed amount can be stated, much depending upon the individual idiosyncracy of the animal. Approximately 75 milligrams of the drug to a 250-gram guinea-pig, and 100 milligrams to a 300-gram guinea-pig produce the degree of hypnosis desired.

We use a fresh 10 per cent. solution of chloral hydrate, carefully measuring out the required amount into a small sterile beaker and adding an equal amount of sterile water. This diluted solution is injected into the muscles of the thigh of the animal, half into one leg and half into the other. If, after 20 to 30 minutes, the needle is inserted into the peritoneal cavity, muscular twitching and slight movement of the head will be noticed. The injection of 5 c.c. serum is then given and the animal kept in a warm room. No symptoms appear and the sleep is undisturbed. After 1½ to 2½ hours have elapsed, the animal slowly recovers from the effects of the drug. No symptoms or ill effects have been observed in any of the animals. Observations have been followed for over two weeks after treatment. The animal, after the effects of the drug have disappeared, will react with characteristic symptoms of anaphylaxis if given a third injection of serum. Up to the present time we have reinjected only 24 to 72 hours after recovery from the effects of the drug.

If the dose of chloral hydrate has not been sufficient, the insertion of the needle into the peritoneal cavity will cause pronounced muscular movements, raising of the head and an attempt to regain its feet. Under these conditions if the serum is injected the animal will die of anaphylaxis.

On the other hand, if the animal shows no muscular twitchings whatever, the dose of chloral has probably been too large. We wish to emphasize the fact that great care must be used not to overdose the sensitized guinea-pig with chloral hydrate, although a sensitized, as well as a normal guinea-pig, will recover from a large dose, considerably more than the amount mentioned above. Apparently the combined effects of the drug and the serum in a sensitized animal produces a deeper hypnosis than the drug when given alone.

Thus far our experiments have been only with the intraperitoneal injection. Besredka's method of injecting directly into the brain will be taken up, and also the method of injecting directly into the vessels, according to Gay and Southard, and into the heart, according to Lewis.

In a number of experiments with calcium chloride, which, according to Besredka, exerts a protective action, we were unable to save the sensitized animals. They died with characteristic symptoms of anaphylaxis.

We have also failed to substantiate Besredka's claims that sensitized guinea-pigs under the influence of ether narcosis are protected from the second injection of ¼ c.c. of serum into the brain. Nor did we find that ether narcosis protected them when the injection was made directly into the vessel or directly into the heart. All the animals died with characteristic symptoms of anaphylaxis in from 2 to 6 minutes. Normal control guinea-pigs under the influence of ether narcosis showed no symptoms or ill effects when subjected to injections of physiological saline solution by the above methods.

In conclusion we wish to add that probably other chemical substances belonging to the same group as chloral will show similar action in protecting guinea-pigs against anaphylaxis.

A STUDY OF THE INTESTINAL FLORA.

Katherine R. Collins, M. D., and Marie Grund, M. D.

(Preliminary Report).

Although the literature shows that much detailed work on intestinal bacteria has been carried on in the last ten or fifteen years, still most of the investigators even within the past few years have worked severally along special lines, and but few attempts have been made to include the many branches of the subject in one work and thus present a comprehensive study of the bacterial flora of the intestinal tract in health and disease. While the main object of the present work is the study of the bacterial causes of summer diarrhoeas in infants, it is believed that the rational way is to precede this by an investigation of the intestinal flora of normal infants under different conditions of diet, etc., and by the study of the intestinal disturbances, complicating other maladies. From this brief outline of the field to be covered, it is perfectly apparent that the present studies which have been pursued for the past three months can not yet have progressed far enough to justify the publication of any data, and this preliminary report is intended merely to define in a general way, the scope of the work to be undertaken.

Considerable difficulty has been encountered in securing from plate cultures returns that seem proportionate to the number and variety of bacteria found in the stained films from fecal material, and some time has been spent in finding suitable media and in evolving a satisfactory technique. Material from infants was obtained chiefly from the obstetric and pediatric wards of the New York Infirmary for Women and Children, and from the dispensary of the same institution. To the time of writing 22 stools have been examined; of these 4 were meconium stools, and 4 were from children suffering from some variety of intestinal derangement. Of the 14 normal children whose stools were examined, 3 were under one month of age, 9 were between 1 and 12 months, and 2 were over 2 years old. Five children were breastfed exclusively, 5 had had breast feeding for some time, but had been fed on cows' milk at least two weeks before examination; 2, under 1 year, were on a milk mixture (malt soup), and 2 had received a mixed diet for some time. Two of the meconium stools produced no growth in the media inoculated, while from one a fine growth of a Gram-positive coccus was obtained, but curiously enough the fourth yielded four bacterial varieties from plate cultures. This is the more striking since, with one exception, the number of varieties isolated from any one stool did not exceed three; in six cases, in fact, only one kind was obtained. Provided the meconium stools are excluded, our tests show that bacilli of the colon type were recovered from all but three of the cases examined.

In the stool of one very young breast-fed infant, smears and cultures showed only the Gram-positive coccus mentioned above, while in two cases the organism obtained was a bacillus which resembles the B. coli morphologically and culturally, except in its action on gelatin. This same bacillus was found associated with bacillus coli and other bacteria in three cases.

Several modifications of the ordinary nutrient agar were employed; besides the various carbohydrates (maltose, lactose, glucose, mannite, etc.), bile agar, beerwort-agar and whey-agar were used. The last-named has yielded rather better results than the others. The method of procedure in general has been the following: One gram of fecal material, obtained as fresh as possible, was rubbed up with 10 c.c. of sterile broth. One loopful of this emulsion was inoculated into a tube of cooled melted agar, and made into plates, which were then grown under anaerobic and aerobic conditions, at incubator temperature from one to three days.

So far, more time has been devoted to the study of infants' stools, but a few adult stools have been examined, and while these have shown a greater variety of organisms, the films made directly from fresh feces, as well as the returns from plate cultures, indicated considerable similarity in the flora of the two kinds of material. It would, of course, be premature to base conclusions on the small number of cases examined. Coccal forms are frequently found in both; a very large, slightly lance-shaped diplococcus, stained by Gram's method, which occurs almost constantly in the adult stools, has been found in about 33 per cent. of the infant cases; and a bacillus of the colon type, encountered in the majority of adult stools, was isolated from about 80 per cent. of the material from infants.

THE PERSISTENCE OF ANTHRAX AND TETANUS SPORES DURING THE PROCESS OF MAKING GELATIN.

KATHARINE R. COLLINS, M. D.

The following experiments were undertaken for the purpose of demonstrating the length of time that anthrax and tetanus spores may persist in infected tissues which have been subjected to the gelatin process:

A calf was infected with a large amount of anthrax spores and bacilli and was then killed. Pieces of the bone and skin were removed and placed in an incubator at 35 degrees C. for twenty-four hours. Examined the next day, these tissues were found to contain large numbers of anthrax spores. The pieces of bone and skin were thoroughly dried and placed in the solutions as below mentioned. The skin and bones of another calf were then prepared by soaking both pieces of bone and of skin in a thick emulsion of tetanus spores. The specimens were then dried and after testing were found to contain many living tetanus germs. These were also subjected to treatment below mentioned.

Tests with Anthrax Spores.

The skin from the infected calf was cut into strips about one-half inch wide, washed thoroughly and subjected to running water for eighteen hours. It was then placed into a lime bath for ten days, which was changed twice during this time. Taken from this bath it was thoroughly washed and all hair removed and then placed again into fresh lime water for four weeks.

At the end of two weeks tests were made and in the smears made directly from the material, well stained spores and bacilli were found. Cultures made from the same strips of skin gave a good growth of anthrax bacilli showing the spores were still alive. At the end of four weeks this same condition still existed. A portion of the skin was then heated in a water bath at 170 degrees F. for thirty minutes and cultures made from this were found to be sterile. Four weeks in the lime bath, together with heating to 170 degrees F. for thirty minutes, was sufficient to kill anthrax spores in the skin.

The bones from this same animal were treated as follows:

They were broken into small pieces, washed with water thoroughly and soaked in a bath of ten per cent. hydrochloric acid for three weeks, the bath being changed occasionally. At the end of three weeks well stained spores and bacilli were found in smears made from this gelatinous substance, and when cultures were made, the spores developed.

After thoroughly washing, the decalcified bone was placed in a lime bath for four weeks, the bath being changed occasionally. After being in the bath two weeks the spores were still active, but at the end of four weeks no growth occurred in the broth inoculated with the gelatinous material.

Some of this gelatinous material was heated, as in the case of the skin, and cultures made, which also showed no growth.

Tests upon bones and skin infected with tetanus spores:

Bones smeared with tetanus spores and bacilli were treated in the same way as the above. No living tetanus bacilli or spores remained alive after two weeks in the lime bath, as in case of the anthrax, although they were still active after being three weeks in the bath of hydrochloric acid.

The tetanus spores attached to the skin were killed after four weeks' exposure to the final lime bath.

These experiments indicate that gelatin derived from infected bones or skin which has been subjected to the above process will not contain living spores of anthrax or tetanus bacilli, if all parts of the tissues are equally exposed to the action of the various baths and to the heat.

RESULTS OF AGGLUTINATION TESTS FOR GLANDERS IN HORSES.

R. E. Pick, M. D., Assistant Bacteriologist.

During the past ten months the sera of 833 horses has been tested for the aggultination reaction for Glanders. These horses have been sent in under the various headings given in table No. 1. These headings bear no relation to the final classification, because it frequently happens that horses apparently normal will give a high agglutination reaction and on being subjected to the mallein test will give a reaction thus indicating the presence of glanders. We have been able to autopsy a few such horses and have found the chaaracteristic lesions of glanders present (see next page).

TABLE I.*

Agglutination.	Suspected.		Exposed.		Normal.		Showing Symptoms of Some Disease.		Clinical Diagnosis, Glanders.		Total.	
	No.	Per Cent.	No.	Per Cent.	No.	Per Cent.	No.	Per Cent.	No.	Per Cent.	No.	Per Cent.
200....................	4	14.81	40	16.00	44	8.80	2	3.92	0	0.00	90	10.91
500....................	3	11.11	54	21.60	101	20.20	10	19.60	0	0.00	168	20.16
1,000....................	5	18.51	60	24.00	118	23.60	14	27.44	1	20.00	188	23.77
2,000....................	9	33.33	55	22.00	104	20.80	10	19.60	0	0.00	178	21.37
5,000....................	4	14.81	22	8.80	78	15.60	7	12.73	2	40.00	113	13.56
10,000....................	2	7.40	19	7.60	55	11.00	8	15.68	2	40.00	86	10.32
Total...........	27	3.24	250	30.01	500	60.02	51	6.12	5	0.6	Total, 833	Total, 100 per cent.

* The percentage in each column is calculated on the total number of animals in that particular class, thus: at 200 dilution we have 4 cases under the suspected division, or 14.81 per cent. of a total of 27 suspected cases. The percentages opposite the totals are calculated on the entire number of animals, namely: on 883.

Table No. 1 is chiefly interesting because it shows first the relation that the agglutination reaction bears to the clinical diagnosis, also that the greater number of cases of all classes react between the dilutions of 1:500 and 1:2000, thus making this range doubtful unless the mallein or clinical symptoms are present. The dilution of 1:1000 shows the greatest percentage of positive reactions, namely, 23 per cent. of the total of all the cases. It will also be noticed in this table that exceedingly few cases are reported with a clinical diagnosis of glanders; in fact, only 5 out of a total of 833 cases, or 0.6 per cent. of the total of cases.

Final reports could not be obtained for all horses tested, but in table No. 2, the results are given in 202 cases. The greatest number of cases discharged or working show an agglutination index between 500 and 2,000, while the number reacting beyond this point diminish. On the other hand, horses reacting to mallein or those dying or destroyed because of glanders show a steady increase in agglutination reaction up to 5,000, with a slight diminution in numbers at 10,000. Even, at this index, however, the number is greatly in excess of those discharged or working, thus only 3 horses out of 34 showing an agglutination reaction of 1:10,000, gave no other evidence of the presence of glanders.

TABLE II.*

Table Giving Agglutinations, and Subsequent Data for 202 Horses in Which Such Data were Obtainable.

Agglutination Reaction.	Discharged or Working.		Mallein.***		Killed or Died from Glanders.		Total.	
	No.	Per Cent.	No.	Per Cent.	No.	Per Cent.	No.	Per Cent.
200.............	7	18.28	0	00.00	3	4.05	10	4.95
500.............	14	24.56	6	8.45	2	2.70	22	10.89
1,000...........	12	21.05	12	16.90	12	16.20	36	17.82
2,000....	13	22.80	20	28.16	17	22.98	50	27.35
5,000....	8	14.03	19	26.77	23	31.08	50	24.75
10,000...........	3	5.26	14	19.71	17	22.98	34	16.83
Total.......	57	28.22	71	35.14	74	36.63	202 Cases.	100

* Table No. 2 is to be read in the same manner as Table No. 1.

This, then, bears out the former conclusion that betwen the range of 500 and 2,000, the evidence of the existence of glanders based upon the agglutination reaction alone is less clear and definite, while the increase in the height of agglutination also increases many times the probability of glanders infection being present. It is also interesting to note that at 500 agglutination, 14 horses were discharged or working and 2 killed or died from glanders out of a total of 22; at 1,000 agglutination 12 discharged or working and 12 killed or died out of a total of 36; or an equal proportion of the two classes; at 2,000 agglutination 13 discharged or working and 17 killed or died, showing a tendency for this degree of agglutination to have some diagnostic value; at 5,000, 8 are discharged or working and 23 killed, showing a marked tendency of the test at this point to have positive diagnostic value.

The number of animals, in the above table, reacting 1:10,000 is hardly large enough to warrant analysis, though it is probable that agglutination at this dilution will have still greater diagnostic value.

THE SIGNIFICANCE AND MICROSCOPICAL DETERMINATION OF THE CELLULAR CONTENTS OF MILK.

Arthur I. Kendall, Ph. D.

The tendency toward segregation of population which has been so marked during the last two decades has necessarily been accompanied by a parallel, retrograde movement of many important industries upon which the inhabitants depend for an essential portion of their daily ration. Perhaps the most noteworthy example of this is the milk supply.

The complex of difficulties which was great under the old regime, where the production was in the immediate neighborhood of consumption has been greatly magnified, and at the same new and intricate conditions have arisen which make the problem of a hygenic milk supply a very difficult one. New and more complete understanding of the laws relating to pure milk and the public health have placed an added responsibility upon the sanitary officials on the one hand to safeguard the populace from careless, ignorant or unscrupulous dairymen and at the same time has made it necessary for them to be able to act in harmony with the producers to enact measures governing the production and distribution.

In order to intelligently fulfill their duty as sanitary authorities, modern methods must be devised which shall become in their hands the tools by which they may determine from time to time the condition of the milk as it is produced, handled and sold.

Such methods need not have, nor, indeed, can they hope to attain the precision of ultra scientific measurements; in fact, proximate methods of analysis in this connection are of greater value; they are more rapid, and permit a greater volume of work in a unit time, a point of the greatest importance where large numbers of purely routine determinations must be made.

Special attention will be paid in this paper to the cellular content of milk-leucocytes (pus cells as they are sometimes called) and bacteria.

The Bacterial Content of Milk.

Bacteria occur in milk from three sources; from the udder tissues itself, from unclean surroundings of the cow, and from additions which may occur at any stage of the handling of milk till it is finally consumed.

Microorganisms in the udder, again, originate in two ways; the first by bacteria which gain access to the milk duct and cistern between milkings, and those that habitually occur in these portions of the secreting tissue. The first class, those derived from outside contamination and which multiply between milkings are, as a rule, harmless in the numbers voided, and vary in type from time to time, depending upon the environment of the cow. The second class also vary, but with a definite animal are apt to become in reality a "physiological flora" which persist in spite of outside conditions. Russell and Moore have found that practically no cow gives milk that is sterile; even the strippings contain a few bacteria, and Moore and Ward have been partially successful in introducing definite species of certain organisms and establishing a semi-permanent flora. It should be noted, however, that bacteria growing in the udder tissue under these conditions is not wholly permanent, but have to be reintroduced from time to time.

Russell has shown that bacteria causing disagreeable taste to milk may become localized in this manner, and at times it is extremely difficult or impossible to remove such growths.

In diseased udders the picture, however, is radically different, and one finds a multitude of bacteria in milk which, under normal conditions would only contain a few bacteria.

Probably the most common organisms occurring in such cases is the streptococcus of mastitis, although Gaffky has isolated several strains of colon-like bacteria which are extremely virulent for experimental animals, and probably not without effect upon man.

The streptococcus of mastitis should be carefully distinguished from the ordinary milk streptococcus, which is very abundant in many samples of milk which have been allowed to stand at temperatures not below 45 degrees C. The former is a variant of streptococcus pyogenes, and has frequently enlarged elements which were called by Hueppe "arthrospores," and usually occurs in long chains. Furthermore the elements are large, usually a micron or more in diameter, while the milk streptococcus is much smaller. The mastitis organism is associated with large numbers of poly-and mononuclear leucocytes, as well as cell detritis, and the whole mass interwoven with strands of fibrin. The intensely blue staining with methylene blue as well as the agglutinated appearance is distinctive.

Bacteria derived from an unclean environment of the cow-dirt, unclean milkers, dirty udders, and those from handling after the milk has left the farm may be almost infinite in their variety, and may contain under exceptional conditions several kinds of pathogenic bacteria.

Estimation of the Number of Bacteria in Milk.

There are two methods by which the number of bacteria may be estimated in milk, the "direct," or smear method, and the indirect method, in which the numbers of colonies developing upon solid nutrient media are taken as an index of the numbers of organisms originally present.

The latter method is inexact for several reasons, party because it is impossible to break up groups or chains thoroughly in the sample; this is so because the milk is very viscous, and there is very little difference in specific gravity between the milk and bacteria; the second cause is the fact that by no means can one with our present methods cause all bacteria to develop in the same medium. The food conditions which are favorable for the majority are unsuited for the minority, and there is no reason, furthermore, for believing that the ratio of those growing to those not growing is constant.

As the result of this difficulty, attempts have been made to find a substitute for the plating method, and the smear method is the direct attempt in response to this need to determine the bacterial content.

There are three general modifications of the direct method; the measure of the sediment (Trommsdorff's method). This is in reality not a bacterial method, but expresses instead the volume of material which can be sedimented from a given volume of milk, and includes not only bacteria and leucocytes, but various other foreign material whose specific gravity is sufficient to bring it down during the process of centrifugalizing.

The Doane-Buckley method corresponds to the direct estimation of blood cells in the blood; the numbers of bacteria and leucocytes which may be counted, when a definite aliquot portion of the milk is placed in a blood counting cell multiplied by a suitable factor furnish evidence of the number of cells originally present.

The third and most practical method is the one first made practical by Stewart, of Philadelphia. The principal feature is the fact that bacteria derived from a definite, representative portion of milk are centrifugalized, the sediment is smeared upon a definite area, and representative definite portions of this area are counted with respect to cellular content, the numbers of cells multiplied by an appropriate factor, and the result gives the number of cells originally present.

Russell has greatly increased the accuracy of the procedure; he noticed that as the cream rises during centrifugalization, varying numbers of bacteria and leucocytes rose at the same time, due to a sort of "raft" action on the part of the fat globules. If, now, one heats the milk to 65-70 degrees C. before the sedimentation, the fat globules become very much less in size, due to a bursting of their envelopes, and there are only a few cells carried up with the fat under these conditions, an amount equal to less than four per cent., as a rule.

Various observers have from time attempted to establish a ratio between the plate count and the direct method, and while these factors may hold with a fair degree of constancy, this must not be taken to indicate in cases of variations from the ratio of duplicate samples in a small number of cases, that one or the other result is necessarily incorrect. A discussion of the reason for this has already been given above.

Pasteurized milk cannot be examined by the smear method for the number of living bacteria, because some of the bacteria killed by heat stain for the most part quite as well as those which have developed, since the pasteurization.

In general, where rapid work and proximate results only are desired, the direct or stained smear method, carefully carried out may, with certain precautions, which will be described in detail in the succeeding portion, be used and relied upon to furnish a fair index of the bacterial content of milk. Good milk shows uniformly a low content in bacteria (except pasteurized samples), while milk bacterially rich can be readily detected.

One of the greatest advantages of this method lies in the fact that samples may be preserved in their original form, without fear of bacterial increase by the addition of a small amount of formaldehyde. Larger amounts of aldehyde, however, cause coagulation of the samples.

Leucocytes in Milk.

The term "leucocyte" has been applied to a variety of cells which may occur in milk, including mono- and polymorphonuclear cells, gland cells, as well as cell detritus in certain instances. Many investigators assume that one cannot distinguish between a leucocyte and a gland cell; this is, however, as a rule, not difficult, because gland cells are, for the most part at least, mononuclear, while the majority of leucocytes are polymorphonuclear. The mononuclear leucocytes, however, may be thus confused.

Although the bacterial content of milk gives one an idea of the present condition of the milk, and in exceptional cases furnish information which may lead back to the dairy, herd or even cow responsible for the abnormality, yet it is extremely desirable to have some factor which shall indicate more specifically the condition of the animal which produced the milk: a factor which does not change after the milk is drawn, and the leucocytes seem to offer such a criterion.

It is evident that they do not multiply after they leave the cow, they are stable toward degeneration and staining reactions for long periods of time and in many ways they seem to be eminently suited for this purpose.

Many studies have been made with the view of determining their significance; attempts have been made to correlate their numbers with the leucocytic or erythrocytic content of the cow's blood, but no such relation can be shown to exist. Stokes and Wageforth showed that cows kept in sanitary conditions were much less likely to have high leucocyte counts in the milk than were those kept in unsanitary surroundings. Text books are silent upon what may be designated the normal or physiological leucocyte content of the blood, and the succeeding pages represent studies made for that purpose. It is known that immediately before and after parturition the numbers may be very high, and legislation has prevented milk from being placed upon the market for a definite length of time after parturition.

Method of Procedure.

Before describing the method employed in this work in detail, it may be well to mention briefly the general plan followed with reference to the collection of samples. Milk was obtained from two farms which furnish "certified milk" of excellent quality to Greater New York. The samples were collected under the immediate supervision of the writer from cows that, so far as careful inspection could show, were undoubtedly normal in every respect. A few samples were taken from animals that had disease also, but these specimens will be discussed under appropriate headings, and are quite distinct from those from normal cows.

To determine the cellular content of milk, samples were carefully and thoroughly shaken, heated to 70 degrees C. for 10 minutes, again thoroughly shaken, and placed in centrifuge tubes of small bore, a cubic centimetre in each, revolved for 10 minutes at 2,400 revolutions, and spread upon slides in areas of two square centimeters each. The smears were then either fixed in the flame, or flooded with a mixture of absolute alcohol and ether, stained with methylene blue, dried and were then ready for examination.

Stewart uses circles having a definite area, and this method is to be preferred to squares of the same size, because the sediment can be distributed easier in a circle than a square, if one is forced to use the stopper of the centrifuge tube as the agent for distributing the bacteria, but for rapid work with many samples the difficulty of establishing accurate circles more than offsets any slight gain in accuracy. If one counts ten representative fields, using a low-power lens as a guide, the results, as shown by duplicate determinations, are strikingly harmonious.

For counting, the writer has used with great success a "net" micrometer, ruled into 100 squares, fitting into the eyepieces at the diaphragm so that the squares seem superimposed upon the microscopic field, and one sees a picture precisely like that seen in a blood counting cell. By regulating the length of draw tube, with the oil immersion lens and a definite eyepiece, it was possible to so adjust the relation between magnification and size of squares so the area counted was exactly 1/10,000 of a square centimetre. Inasmuch as the contents (cellular), of a cubic centimetre were spread upon two square centimetres, it was possible, by multiplying the average number of cells per field by 20,000 to determine with reasonable accuracy the number in the original sample.

The sources of error are:

1—Error in sampling.

2—Error in spreading on the slide.

3—Loss of cells by washing off, due to imperfect fixation methods.

4—Error of counting.

The error due to imperfect fixation is variable; careful comparisons of samples fixed with absolute alcohol and ether with the flame showed practically no difference. On the other hand, in almost every instance in which there were fairly large numbers of cells per field (70–200), examination of the water which was used to replace and remove the excess of stain showed a variable number of organisms; slides stained with methylene blue, but in which the stain was allowed to become dry, and examined without washing showed in such cases a rather higher content, and this observation is in accord with that of Hehewerth, who found a large percentage of bacteria removed by washing when they were fixed in the ordinary way. The losses in the milk slides, however, were much less than those reported by Heheworth, and this is undoubtedly due, in part at least, by the "fixatif" action of the milk proteids.

The spreading error has been commented upon, and there is no method of overcoming it except by a careful examination with a low power lens preparatory to counting, and rigid attention to the distribution in making the determinations.

The greater the total area counted, other things being equal, the greater accuracy the method; in practice, it is hardly possible to count more than ten representative fields, using the net micrometer, but experience has shown that agreement between averages so obtained in duplicate samples is rather less variable than those obtained by plating methods on the average. If, however, there are very few or very many cells per field, the agreement is less close, but here again there is parallelism with plate cultures. Where there are very few or very many colonies.

The advantages of the method are:

1. Both leucocytes and bacteria are counted. Frequently one may obtain valuable information about the past history of the milk and the cow which the bacterial count alone would utterly fail to detect.

2. Samples may be preserved in their original state by the addition of formaldehyde with no further increase of bacteria.

3. Examinations may be made the same day that the samples are taken, finished and recorded.

4. A rather definite idea of the condition of the dairy, herd or cow, as the case may be, may be obtained in daily samples; the exact value, however, of this information is at present uncertain.

5. A general idea of the presence or absence of certain types of bacteria present may be determined; this is of importance in many connections.

6. A much greater number of samples can often be analyzed by the smear method than are possible with the plating method, taking into consideration the preparation of media, sterilizing apparatus and space required.

7. While there is no means at present available to determine the accuracy of either the plating method or smear method, duplicate determinations made with the latter procedure as a rule vary within 20 per cent., provided there are not more than 70 per field, which is as close as is possible with bacterial methods in general.

The Leucocytes of Normal Cow's Milk.

Milk freshly drawn from 168 cows was examined by the smear method as described above, and over 80 per cent. showed less than 400,000 leucocytes per cubic centimetre of milk, or on an average of less than 20 per microscopic field. These cows were as far as a rigid inspection could show, free from any udder disease, and were normal in every respect. The fore milk, generally speaking, contained relatively a few less leucocytes than the strippings, but this difference was not marked nor constant. This fact is of importance in determining the numbers of leucocytes that may be allowed in market milk; the present legal limits in those laboratories which have made careful studies of this subject are in close accord with this number, Boston, for example, insisting 500,000 or less as the permissible number.

What can be done in this respect by dairymen is shown by the following table: the numbers of leucocytes per field, multiplied by 200,000 will give the number per cubic centimetre.

A herd of 28 cows were chosen; they were in perfect health, very carefully groomed and hygienically housed.

Leucocytes Per Field.	Number of Cows.
1–10	22
11–20	2
21–30	2
31–50	2
above 50	none.

The period of lactation, that is, the time elapsing since the cow calved, seemed to make absolutely no difference in the leucocyte content, provided the samples were taken at least 10 days after parturition, after the cow had again become a normal producer. Cows that had been milked continuously for periods up to a year, and those that were only recently milked showed absolutely no difference in this respect. This statement is based upon the findings of the 168 cows referred to in Table I., and comparisons with a few cows from other herds seem to substantiate this statement.

TABLE I.

Table Showing the Numbers of Leucocytes per Field in the Milk of Normal and Discceased Cows.

Number Leucocytes per Field.	Numbers of Cows in Each Class.					
	Normal.	Tubercle.	Three Teaters.	Garget.	City Milk.	Cows—Days After Calving.
0-10	102	25	25	None.	49	6
11-20	36	7	13	None.	11	2
21-30	9	5	9	None.	5	1
31-50	10	9	16	None.	5	1
51-75	8	9	3	None.	4	5
76-100	3	4	3	None.	5	3
101-150	..	1	7	2	4	5
151-200	..	1	8	1	2	..
201-300	..	2	5	3	2	1
......	168	63	89	6	87	24

Market milk cannot, theoretically, be compared directly with these statements; milk retailed in cities is obtained from a large number of cows, and one poor cow (high in leucocytes), would, generally speaking, make very little difference if that milk were mixed with the milk of a dozen normal cows. At the same time, the milk will attract attention, even in this case because the agglutinated appearence still persists, and one is very apt to find clumps of leucocytes, many of which may be phagocytic, which at once attract attention.

A few of the cows showed a rather high leucocyte content, in spite of the fact that they were apparently normal. The exact significance of this phenomenon is at present a mystery, and so far the writer has been unable to offer any definite explanation. It is possible that by carefully watching such animals, some infection might be noticed. It is interesting to note in this connection the fact that anything that tends to seriously disturb the animals may result in a definte increase in the numbers of leucocytes. The writer happened to be in a barn in which the animals were being tested with tuberculin; previously the cows had shown moderate counts, as a rule below 20 per field; at the time of the testing, the same animals (27), showed in over 80 per cent. of the cases a decided increase in leucocytes, not dependent upon the presence of a positive reaction, but apparently due to the unusual disturbance. After a week, those animals which were again examined, were back to their normal.

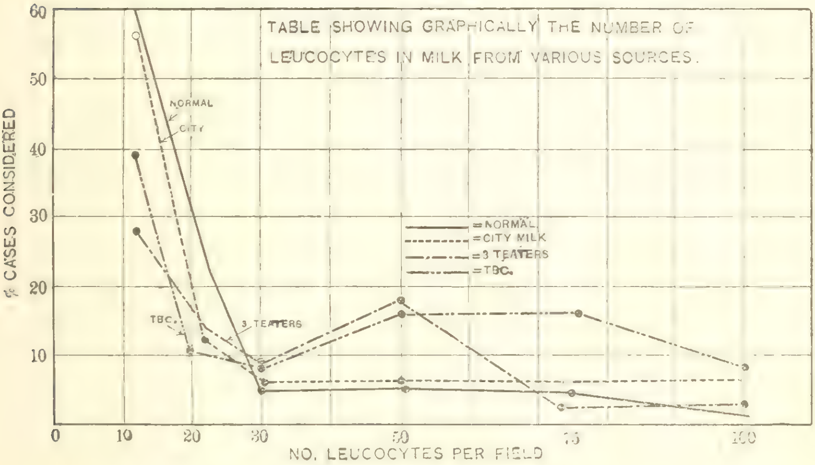
Milk from Cows Reacting to the Tuberculin Test.

The results are not as satisfactory as the preceding ones, because it was impossible to observe the cows at the time of milking to detect signs of udder disease which might influence the results, except in a few cases. In every sample, however, an tubercle bacilli could be detected by direct staining with the customary Ziehl-Neelson stain, or by the guinea pig test, using the sediment from 10 c.c. of milk. Table 1 shows the leucocytic content; the variations are greater by far than was the case with the normal cows. In certain of the samples it was possible to correlate the high leucocyte count with the presence of streptococci; the samples were fresh, and this fact, coupled with the fact that the cells were in many instances phagocytic, probably explains some of the abnormal results.

"Three-teaters."

Cows known as "three-teaters" have been the subject of discussion for some time; the trend of opinion has been to exclude them from the herd, and the results obtained in this work, based upon the examination of 89 cows certainly shows that in relatively large proportion of such animals there are an usually high number of leucocytes (see Table II.). The leucocytes are frequently phagocytic, and in many cases associated with streptococci, rarely with bacilli.

Table II.



It is interesting to note in this connection that Kitt, Guillebeau, Lucet and Streit have isolated colon-like bacilli from cases of mastitis; in some cases recovery was attended, apparently by occlusion of one or more teats. The organisms agreed in important details, furthermore with the colon bacillus, and it is extremely suggestive to compare these results with those of Jackson and others who have found colon bacilli by presumptive tests in milk, obtained from the city supplies.

In general, while a rather large proportion of three teaters may show abnormal numbers of leucocytes in their milk, it would be an injustice to arbitrarily exclude all such animals from the herds by law. Over 40 per cent. of the cases reported in this paper were quite as normal in this respect as the well kept cows of excellent dairies. It is not difficult to determine whether or no the inflammatory process, in those animals which have suffered from mastitis, had disappeared; and certainly congenital three-teaters have no inherent abnormalities, except a lack of complete development in the udder.

City Milk.

In all, 87 samples of city milk were examined in order to determine how, in general, mixed milk from a great variety of sources compared with milk from normal, individual cows. In no sense was the milk obtained for this purpose; it represents samples precisely similar in every respect to those sold every day in New York from the smaller shops and stores in small amounts.

By referring to the table it will be seen that about 66 per cent. compares very favorably with that derived from normal cows; where the animals are carefully housed and groomed. At the same time there is rather a large proportion which is not so clean. In the sediments one may actually see bits of hair, pieces of manure and amorphous matter. When one realizes that there is a possibility that such milk was made up of a few bad samples with a larger proportion of good milk, the significance of the microscopical examination in such cases becomes patent. In such milk as would under such conditions contain from 100 to 200 leucocytes per field, there are almost always very striking indications of abnormalities on the part of the cow; phagocytic cells are not rare, one sees streptococci of the larger types and in general there is a much more intensely staining reaction.

The bearing of this work upon the control of municipal supplies is the chief raison d'etre for its initiation, and if the results which are represented above are not influenced by seasonal factors which, of necessity, could not for obvious reasons be taken into account, there is reason to believe that it is feasible to require dairies to furnish milk containing not more than 400,000, or at the most, 500,000 leucocytes per cubic centimetre. That over 80 per cent. of the cows in well-kept farms make this record is not new, and even the fact that a large percentage of regular city milk is of this quality shows that such a standard would not be a very great hardship to place upon the producer.

Summary.

1. The smeared sediment method of examining milk is rapid, practical and reasonably accurate.

2. Certain precautions must be employed in making such determinations.

3. Normal cow's milk in over 80 per cent. of the cases examined showed less than 20 leucocytes per microscopical field, or 400,000 per cubic centimetre.

4. This number of leucocytes is proposed as the legal limit in milk sold for human consumption.

5. Cows having mastitis or other udder disease should be rigidly excluded from herds, not only because they give diseased milk, but because there is danger of the disease being transmitted from cow to cow by milking.

A COMPARATIVE STUDY OF THE DIRECT MICROSCOPICAL AND PLATING METHODS FOR THE BACTERIOLOGICAL EXAMINATION OF MILK.

By Harriet L. Wilcox.

The following work was undertaken in order to compare the smear with the plate method for the bacteriological examination of milk. Of the 183 samples examined, all with one or two exceptions were sent in by the Department of Health Milk Inspectors and regarded as "ordinary milk."

The technique used was essentially that of Stewart, and was as follows: After the milk had been thoroughly shaken fifty times, one c.c. was pipetted off from each sample and placed in a small glass tube, three inches in length and one-quarter of an inch in diameter and closed at either end with rubber corks. The tubes were then centrifugalized for 10 minutes, after which the upper cork was removed and the supernatant milk and cream were gently poured off. The lower cork upon which the sediment had been precipitated was then removed, and rubbed as evenly as possible on a slide over an area of 2 square centimeters upon which a drop of sterile water had been previously placed. The smears having been allowed to dry in the air were fixed with methyl alcohol, and stained for a few seconds in a watery solution of methylene blue.

It was found that there was no loss in the milk smears when the slides were fixed in this way, while in every case where the smears were fixed by heating, which necessitated washing the slides to remove the excess stain, the wash water was seen to have a decidedly milky appearance, and smears made from it showed that a considerable percentage of bacteria had been washed from the milk smears.

Russell found that by heating the milk from 65–70 degrees C. the fat globules were diminished in size, due to the bursting of the envelopes and that only a few cells were carried up with the cream during centrifugalization; it was thought that this would hold true in regard to the bacteria, so that from each sample examined duplicate smears were made from a portion of the milk which had been previously heated from 65–70 degrees C. for 10 minutes.

To estimate the number of bacteria present, ten fields of each smear were counted, averaged, and multiplied by 20,000, inasmuch as the net micrometer used in conjunction with the 1/12 oil immersion gave a field equal to 1/10,000 of a square centimeter, and that the bacterial content of one cubic centimeter was spread over an area of two square centimeters. Two counts were made from each smear, in the first the bacteria, if in clumps, were regarded as such, while in the second the number of individual organisms was estimated as nearly as possible. The two counts made from the smear were then compared with the bacterial count obtained by plating.

TABLE I.

Unheated Milk (direct count).			Milk Heated from 65°-70° C. (direct count).		
Number of Samples.	Bacteria (group).	Bacteria (Indiv.)	Bacteria (group).	Bacteria (Indiv.)	Colonies in Nutrient Agar, Plated.
197	2,316,000	8,820,000	420,000	4,134,000	7,360,000
1,152	1,746,000	6,018,000	966,000	5,268,000	5,240,000
1,965	[illegible]	11,976,000	330,000	3,702,000	3,870,000
1,249	3,252,000	9,192,000	360,000	2,088,000	2,670,000
22	7,596,000	31,740,000	900,000	5,502,000	6,000,000
1,955	7,536,000	18,828,000	1,074,000	21,732,000	5,844,000
231	3,120,000	13,002,000	312,000	4,644,000	3,760,000
1,858	2,364,000	6,294,000	186,000	1,944,000	2,950,000
[illegible]	3,384,000	22,680,000	1,032,000	11,862,000	4,600,000
1,860	2,586,000	24,444,000	948,000	17,076,000	9,700,000

It being impracticable to tabulate the counts of all the milk examined, the above ten samples were chosen as representing fairly the total results obtained.

By a comparison of the results obtained by direct method with those of plating (Table I.), it is difficult to draw any conclusions as to which is the more valuable method. In some milks where the bacteria are well distributed and not in chains or clumps the individual bacterial counts from the smears compare very closely with those from the plates, while in other milks the group bacterial counts agree much more closely with the plate counts and it is just here that frequently a great inaccuracy in milk plating is strikingly brought out, *e. g.*, the number of colonies in a milk plate is regarded as representing the number of bacteria in a given sample of milk; in some milks, however, the organisms are held together in chains or masses which are not easily broken up by shaking, and a colony may, therefore, represent a group composed of from one to two hundred or more bacteria, thus giving an erroneous estimate of the actual number present.

On the other hand, in counting only a limited number of fields in a smear, there may be as great a discrepancy in the resulting counts as in milk plating. For example, the following duplicate counts were made from five smears, the fields in both counts being taken as nearly as possible from the same part of the smear.

Number of Samples.	Bacteria.	
	Original Count.	Duplicate Count.
467	38,000	272,000
605	104,000	270,000
382	642,000	338,000
397	32,000	152,000
543	532,000	510,000

An advantage of the smear method is that it is possible to estimate not only the number, but to some extent the different varieties of organisms, within two hours after the arrival of the milk at the laboratory.

In regard to heating the milk to 70 degrees C. for 10 minutes before centrifugalization to obtain a higher and more accurate count of the bacteria present the results have been most disappointing. The comparison of the counts made from the heated samples of milk with those of the unheated shows that in 25 per cent. we get a higher bacterial count on the average in the unheated milk (see Table I.). Although a comparative count of the leucocytes was not made, a great increase in their number was very noticeable in those smears made from the heated milk.

To determine just what proportion of bacteria in one cubic centimeter was thrown down by sedimentation, the samples of milk were centrifugalized after which plates were made from the cream, middle portion and sediment respectively. The plates were then incubated at 27 degrees C. and counted after 72 hours. The results showed that the bacterial count of the cream and middle portion together was, on the average, twice as high as that from the sediment alone.

For routine work it would scarcely be practicable to count the individual organisms in each smear, but after a little training one could estimate approximately the number of bacteria present. If the smear showed on the average two or less organisms per field, the bacterial count for that sample would be less than 50,000 organisms per c.c., and might, therefore, be considered of good quality. When the microscopic field was filled with a hundred or more bacteria or large clumps comprised of several hundred bacteria, it is clearly evident that the milk contains 1,000,000 or more organisms per c.c., and would be regarded as poor milk. The smears from milk between these two extremes, however, might require a more accurate counting, or it might even be advisable to plate.

Conclusions.

1. The smear or direct method for examining milk as it takes but a few hours is much more rapid than the plate method.

2. The determination of the different morphological varieties of organisms and also the cellular content of the milk is possible by this method.

3. To make a leucocytic count the milk should be heated to 70 degrees C. for ten minutes; for bacterial count, the raw milk gives better results.

4. After centrifugalization for ten minutes, about one-third of the total number of bacteria in one cubic centimeter were thrown down, the rest being suspended in the supernatant milk and cream. The bacteria seen in the smear represent, therefore, only about 33 per cent. of the total number of the bacteria in the milk.

5. For routine work, it is not necessary to make an accurate count of the bacteria, but merely to examine the slide as a whole and estimate the number present.

6. That by employing either the plate or smear method the results thus obtained are only approximate.

THE TIME AND TEMPERATURE FACTORS IN THE BACTERIOLOGICAL EXAMINATION OF MILK.

Harriet L. Wilcox.

I. *Temperature Most Advantageous for the Growth of Milk Bacteria.*

There are varied opinions among laboratory workers as to what is the most suitable temperature for the incubation of milk plates. Some believe that the maximum growth of the more important bacteria is obtained when the plates are grown at 37 degrees C., while others are in favor of room temperature, as giving a more reliable count. To determine this point as nearly as possible, plates were made from certified and ordinary milk and were grown at 37 degrees C., 27 degrees C. and room temperature, the latter averaging about 23 degrees C. The number of bacteria in the duplicate plates grown at the above temperatures was counted and averaged, the plates incubated at 37 degrees C. and 27 degrees C., being counted for four consecutive days, while those grown at room temperature were counted from the second to the seventh day, inclusive. In the 17 certified milk samples examined, 10 or 58 per cent. showed on the average twice as high a count when grown at room temperature (Table I.); the results in the ordinary milk were even more striking, 52 per cent. of the samples having shown on the average nine times the number of organisms in the plates grown at room temperature than those at 37 degrees C. (Table II.). In the second experiment, ordinary market milk alone (both unheated and heated), was used and plates made from it were grown at 37 degrees C., 27 degrees C. and room temperature. The maximum growth was obtained at 27 degrees C., in 65+ per cent. of the samples tested, while those at the room temperature had the greatest number of bacteria in 26 per cent., thus leaving only a small proportion having the maximum growth at 37 degrees C. (Table III.). The two samples showing a slightly greater number of colonies at 37 degrees were "pasteurized" milk. This previous heating in destroying the non-spore bearing varieties undoubtedly was the cause of this variation in these samples from all of those not so treated.

II. *Summary and Conclusions Upon the Time and Temperature Most Suitable for the Bacterial Count of Milk.*

In the appended tables it will be noticed that in every case the highest count is at the end of the fourth day or later, without regard to which temperature was used. The difference, however, is not sufficient to make it advisable to delay beyond a certain length of time. It is noticed that at 37 degrees there is a marked increase in the number of colonies at the end of the second day over the first, and then only a moderate increase for each successive 24 hours. Probably all would agree that when subjected to 37 degrees, 48 hours is a sufficient time to allow for growth. It is true that another day will allow of something like 10 per cent. additional growth, and a fourth day another slight increase, but this is too little to make such a delay advisable.

At 27 degrees there is also a great increase between the count at the end of 24 hours and at the end of 48 hours. There is a still considerable increase in the next 24 hours, and a smaller one at the end of the fourth day. Probably most would choose either a growth for 48 hours or for 72 hours. Where the delay is not important 72 hours should be regarded as the best time to allow for a growth. Here, as at 37 degrees, it is to be remembered that a considerable increase will take place if a longer time is allowed for the development of colonies. At 23 degrees there is even a greater difference between the growth at the end of 24 hours and at 48 hours, and a slighter greater difference at the end of 72 and 96 hours. Here, probably, most would agree that either a three-day or four-day period of growth be allowed, three days giving a practical time limit in our opinion.

When we consider the temperature at which milk bacteria should be allowed to develop, the enormous difference between the counts of colonies developing at 37 degrees and at 27 degrees or lower is at once apparent. At the lower temperatures from ordinary market milk from two to ten times as many colonies develop, and from purer milk a slightly less excess. It seems to us, therefore, hardly to be open to argument that one of the lower temperatures should be advised. Between 27 degrees, 23 degrees and still lower temperatures down to 20 degrees there seems to be but little choice. If it is desirable to have always the same temperature we should advise one of 27 degrees, because this is easy to obtain the entire year and also because it gives a somewhat more rapid growth. It seems, however, justifiable from our results to allow the colonies to develop at any temperature between 23 degrees and 27 degrees, simply allowing a day longer for those growing at 23 degrees than those at 27 degrees.

TABLE I.

Certified Milk. Name of Sample.	Average Counts from 2 Plates Grown at 37° C.	Average Counts from 2 Plates Grown at R. T., 23° C.
R. G. F.	1,800 colonies per c.c.	1,550 colonies per c.c.
Meadowedge	350 " "	700 " "
Gedney	2,450 " "	350 " "
Brookside	800 " "	1,250 " "
White Clover	4,250 " "	6,300 " "
Eralaust	250 " "	1,350 " "
Gedney*	250 " "	250 " "
Meadowedge	8,500 " "	5,900 " "
Brookside	1,600 " "	1,200 " "
Briarcliff	5,150 " "	1,800 " "
M. M. F.	10,200 " "	11,050 " "
K. G. F.	1,200 " "	2,250 " "
Brookside	250 " "	700 " "
Briarcliff	3,500 " "	4,200 " "
Gedney	1,650 " "	2,250 " "
Locust	21,550 " "	67,500 " "
Thorndale	1,100 " "	650 " "
Total	64,850 " "	109,250 " "
Averages	3,814+ " "	6,426 " "

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* This sample is disregarded in the calculations as the counts at the different temperatures are the same.

TABLE II.

Ordinary Milk. Number of Samples.	Average Counts from 2 Plates Grown at 37° C.	Average Counts from 2 Plates Grown at R. T. 23° C.
204	80,000 colonies per c.c.	5,000 colonies per c.c.
878	20,000 " "	40,000 " "
1,383	20,000 " "	35,000 " "
1,521	55,000 " "	15,000 " "
1,557	55,000 " "	20,000 " "
1,286	120,000 " "	40,000 " "
1,334	160,000 " "	100,000 " "
1,705	25,000 " "	20,000 " "
1,892	45,000 " "	55,000 " "
2,028	20,000 " "	75,000 " "
707	25,000 " "	30,000 " "
737	230,000 " "	615,000 " "
988	455,000 " "	70,000 " "
1,516	3,370,000 " "	995,000 " "
1,891	30,000 " "	20,000 " "
934	245,000 " "	2,655,000 " "
1,051	15,000 " "	575,000 " "
1,128	60,000 " "	175,000 " "
1,281	360,000 " "	5,250,000 " "
Totals	5,390,000 " "	10,790,000 " "
Averages....................	283,684 " "	567,894 " "

TABLE III.

Counts from Plates Grown for Four Days.

Ordinary Milk. Number of Sample.	Counts from Plates Grown at 37° C.	Counts from Plates Grown at 27° C.	Counts from Plates Grown at Room Temp.
	Colonies per c.c.	Colonies per c.c.	Colonies per c.c.
847	60,000	705,000	385,000
1,097	100,000	325,000	135,000
1,200	415,000	7,495,000	6,800,000
1.400	1,200,000	10,220,000	10,590,000
1,596	255,000	1,375,000	790,000
356	210.000	405,000	270,000
696	2,425,000	8,690,000	6,510,000
*992	65,000	150,000	140,000
*1,014	85,000	45,000	50,000
942	2,245,000	5,030,000	11,295,000
1,346	3,440,000	12,115,000	7,675,000
1,658	60,000	3,170,000	3,250,000
2,019	2,000,000	2,910,000	2,735,000
*362	40,500	18,000	26,000
*1,034	2,500	21,000	16,000
*1,142	11.500	18,500	16,000
*1,151	8,000	19,500	31,000
*1.199	14,500	31.500	28,000
918	155,000	235,000	170,000
1,152	300,000	1,265,000	1,930,000
1,191	365,000	2,810,000	3,335,000
1,286	220,000	2,990,000	2,855,000
1,924	100,000	2,080,000	1,880,000
Totals..	13,770,000	62,912,000	60,912,000

* Pasteurized Milk.

Taking the number of colonies obtained at 27 degrees C. as a basis it was found that 98 per cent. of the colonies developed at 23 degrees C. as compared with 22 per cent. of colonies developing at 37 degrees C.

TABLE IV.

Certified Milk; Dilution, 1-100.	37°C. Colonies per c.c. 1st Day.	2d Day.	3d Day.	4th Day.	23°C. Colonies per c.c. 1st Day.	2d Day.	3d Day.	4th Day.	5th Day.	6th Day.	7th Day.
Brookside...........	175	250	250	250	Not counted.	300	450	450	450	700	700
Briarcliff............	2,650	3,250	3,450	3,500	..	2.150	3.250	3,450	3,900	3,900	4,200
Gedney.............	900	1,400	1.450	1.650	..	850	1.650	1,800	2,000	2.100	2,250
Locust..............	10,900	16,150	20.000	21.550	..	48.650	58.600	60.050	62,800	65.850	67,500
Thorndale..........	650	900	1.000	1,100	..	250	400	450	600	600	650
R. G. F.............	750	1,600	1.800	1,800	..	250	450	Sunday.	1.100	1.450	1,550
Meadowedge	200	250	300	350	..	125	350	..	600	650	700
Gedney.............	950	2,150	2.400	2,450	..	75	150	..	250	300	350
Brookside...........	550	700	700	800	..	150	600	..	1.000	1,100	1.250
White Clover........	1,700	3,100	3.950	4.250	..	250	1.950	....	5,300	6.000	6.300
Brookside...........	900	1.550	1,600	1,600	....	300	350	....	1,000	1.100	1.200
Briarcliff Herd......	3.250	4,700	4,950	5,150	....	100	750	....	1,450	1,800	1,800
M. M. F............	8,250	10,000	10.200	10,200	....	2.500	7,750	....	9.750	10.750	11.050
Meadowedge	4,800	7,500	7,900	8,500	....	1.100	2.300	....	4,200	5.250	5.900
R. G. F.............	550	1,000	1,200	1,200	....	900	1,450	....	2,150	2,200	2,250
Totals.........	37,175	54,500	61,150	64,350	....	57.950	80,450	{ 87,000 Est. }	96,550	103,750	107,650

TABLE V.

Ordinary Milk. Number of Samples.	37° C. Dilution = 1/10,000.				27° C. Dilution 1/10,000.				23° C. Dilution = 1/10,000.			
	Average Counts.				Average Counts.				Average Counts.			
	Colonies per c.c.				Colonies per c.c.				Colonies per c.c.			
	1st Day.	2d Day.	3d Day.	4th Day.	1st Day.	2d Day.	3d Day.	4th Day.	1st Day.	2d Day.	3d Day.	4th Day.
847	30,000	60,000	60,000	60,000	130,000	635,000	670,000	705,000	55,000	315,000	375,000	385,000
1,097	30,000	75,000	100,000	100,000	105,000	270,000	325,000	325,000	1,000	105,000	120,000	135,000
1,200	235,000	395,000	405,000	415,000	2,945,000	7,080,000	7,445,000	7,495,000	2,125,000	6,110,000	6,675,000	6,800,000
1,400	675,000	1,005,000	1,105,000	1,200,000	4,085,000	9,060,000	9,915,000	10,220,000	2,460,000	8,705,000	9,535,000	10,590,000
1,596	75,000	145,000	230,000	255,000	395,000	890,000	1,325,000	1,375,000	45,000	610,000	710,000	790,000
315	15,000	85,000	90,000	90,000	60,000	150,000	205,000	240,000	77,500	135,000	150,000	175,000
872	15,000	45,000	50,000	55,000	45,000	100,000	110,000	120,000	5,000	75,000	105,000	115,000
1,025	5,000	55,000	65,000	70,000	15,000	70,000	155,000	180,000	5,000	75,000	130,000	175,000
1,782	30,000	30,000	30,000	40,000	15,000	30,000	60,000	70,000	15,000	25,000	35,000	45,000
2,019	40,000	50,000	50,000	55,000	5,000	15,000	15,000	20,000	5,000	30,000	45,000	70,000
942	Holiday.	1,530,000	2,005,000	2,245,000	Holiday.	4,530,000	4,800,000	5,030,000	Holiday.	9,010,000	10,530,000	11,295,000
1,346	"	3,040,000	3,180,000	3,440,000	"	11,005,000	11,860,000	12,115,000	"	6,470,000	7,335,000	7,675,000
1,658	"	40,000	55,000	60,000	"	2,883,000	2,905,000	3,170,000	"	2,885,000	3,175,000	3,250,000
1,958	"	2,935,000	3,090,000	3,270,000	"	13,630,000	14,370,000	15,630,000	"	14,025,000	16,810,000	17,680,000
2,019	"	1,925,000	1,930,000	2,000,000	"	2,785,000	2,850,000	2,910,000	"	2,590,000	2,715,000	2,735,000
Total counts of the first ten samples for the four days.......	1,150,000	1,945,000	2,185,000	2,340,000	7,800,000	18,300,000	20,225,000	20,750,000	4,793,000	16,185,000	17,880,000	19,280,000
Total counts of fifteen samples for the four days, the counts of the last five samples being estimated not counted for the first day................	6,930,000	11,415,000	12,445,000	13,355,000	22,320,000	53,135,000	57,010,000	59,605,000	15,150,000	51,165,000	58,445,000	61,915,000

REPORT OF THE BACTERIOLOGICAL EXAMINATION OF A TYPHOID CARRIER.

By Dr. Mary E. Goodwin and W. Carey Noble.

On March 20, 1907, Mary M., a cook, was sent to the Detention Hospital to have her feces and urine examined for typhoid bacilli. She was suspected by Dr. Soper[1] of being the cause of twenty-four cases of typhoid fever in nine families for whom she had worked during a period of five years. The patient seemed to be perfectly well and denied having had typhoid. Her statement, however, is open to question.

On her admission, Conradi-Drigalski plates were made from her feces, and agar plates and broth cultures were made from her urine. The following day the Conradi-Drigalski plates showed about 80 per cent. typhoid-like colonies. Twenty-five of these colonies, which were all the colonies there were in a small area, were fished into broth and tested for agglutination with a high-grade anti-typhoid horse serum. All seemed to be the same organism and agglutinated in dilutions of 1–10,000, 1–20,000 and 1–40,000, while the controls remained actively motile.

Two of these cultures were carried out on the following media: broth, slant agar, stick agar, litmus milk, glucose-peptone-water fermentation tubes, neutral-red lactose peptone water fermentation tubes, gelatin stab, and Dunham's peptone solutions, tests for indol being made on the third and the seventh days. On all media their behavior was the same as that of the laboratory typhoid culture (Mt. S.), used as control. The following tables show the results of the agglutination tests compared with those of the laboratory typhoid culture and a laboratory colon culture.

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[1] Journ. Amer. M. Ass. 1907-XLVIII, 2019-2022.

Table I.

	Antityphoid Horse Serum.			Mary M.'s Serum.		
	1-10,000	1-20,000	1-40,000	1-100	1-200	1-500
Mt. S.	++	+1	+	+	+	—
Mary No. 1	++	+1	..	±	±	—
Mary No. 2	++	++	+1	++	+	±
Colon	—	—	—	—	—	—

++ = Absolute agglutination.
+1 = Complete.
+ = Agglutination.
± = Fair agglutination.

Table II.

Absorption of Agglutinins.

	Antityphoid Horse Serum. Absorption by Mt. S.		Antityphoid Horse Serum. Absorption with Mary No. 2.	
Dilution of Filtrate.	1-100.	1-200.	1-100.	1-200.
Mt. S.	—	—	—	—
Mary No. 2	—	—	—	—
Colon	—	—	—	—

The agar plates and broth cultures inoculated with urine showed no typhoid. Three more specimens of urine were taken later and examined as before, but no typhoid bacilli were found.

The feces have been examined on an average of three times a week from March 20, 1907, to November 16, 1907. In only a comparative few did we fail to find the bacilli. During the summer months, July, August and September, we noticed that the plates contained only a few typhoid-like colonies, about one or two. In July five consecutive negative tests were followed by one showing about 40 per cent. typhoid-like colonies. During August, the stools showed no typhoid. In early September we began to find typhoid again, but only one or two colonies appeared on a plate. From September 11, 1907, to October 14, 1907, the feces again failed to show any typhoid bacilli. During this time the patient's diet was carefully regulated and she was receiving mild laxatives. On October 16, 1907, we made a very thorough test of the feces.

Six broth tubes were inoculated with the feces and from each of these three Conradi-Drigalski plates were inoculated. The following day all the eighteen plates showed about an equal number of typhoid-like colonies, each containing about 75 per cent. Thirty-six fishings were made into broth and tested with antityphoid horse serum in dilutions of 1–10,000 and 1–20,000. All gave a complete agglutination in both dilutions, while the controls remained actively motile.

Since October 16, 1907, weekly examinations of the stools have been made up to February 5, 1908, and with only two exceptions we have found from 25 per cent. to 50 per cent. typhoid-like colonies on the plates. These two exceptions were on November 13, 1907, and December 4, 1907, when no typhoid was found. On February 5, 1908, the last examination made up to present date, the plates contained fully 95 per cent. typhoid-like bacilli.

Urotropin has been given to the patient in 7-grain tabloids three times a day for the last three weeks; that is, since January 18, 1908. The three stools examined since the beginning of this treatment have all contained large numbers of typhoid bacilli.

A typhoid culture from Mary M. was tested for its resistance in sterile Croton tap water. It lived six days. On April 6, 1907, when we obtained almost a pure culture of typhoid from the stool, we inoculated the earth out of doors with some of the feces. The spot was carefully protected, but the following day the plates inoculated with the soil failed to show any typhoid bacilli.

Literature (*typhoid*).

1. Von Drigalski and Conradi H. in "Zeitschr. f. Hyg. und Infect.," vol. 39, 1902, pp. 281-300, state that they found typhoid bacilli in the stools of four persons who had no typhoid symptoms, but were in contact with typhoid patients.

2. Koch, R., in "Die Bekämpfung des Typhus," in "Veröffentlichungen aus dem Gebiete des Militär-sanitätwesen," pp. 20-24, says that in 32 cases of typhoid in which the stools were carefully examined and the organisms carefully isolated he failed to find typhoid bacillus in a single case after three months. He considers the typhoid bacillus an obligatory parasite for man and believes that water-borne typhoid is very much overestimated.

3. Frosch, B., in Festschrift zum sechzigsten Geburtstag von Robert Koch, pp. 691-703, suggests that houses in which cases of typhoid developed one after another where there seem to be no traceable outside sources of infection contain, in all probability, typhoid carriers. He does not, however, prove this by the examination of stools.

4. Dönitz, W., in Festschrift zum sechzigsten Geburtstag von Robert Koch, pp. 297-314, states that in tracing the source of infection in 122 cases of typhoid reported in Berlin during a period of six months, 39 of which proved not to be typhoid, 37 were due to direct contagion or to infected water or milk, and 83 were of doubtful origin. Included in the 83 he reports the following cases:

(a) A woman had typhoid after a visit in the country. In November she was dismissed from the hospital as cured. The following June her husband was taken sick with typhoid. An examination was then made of the wife's urine and typhoid bacilli were found. It was assumed, therefore, that they had been present during the entire seven months and had finally led to the infection of her husband. During this entire interval the woman was seemingly healthy.

(b) A similar case occurred in Basdorf. Two children were apparently infected by the mother who, though not sick herself, proved to have typhoid bacilli in her urine. It should be stated, however, that the surroundings were not clean, and that four months earlier there had been a case of typhoid on the premises. It was from this case that the mother became infected. Typhoid bacilli were also isolated from the ground in the yard where feces had been thrown.

5. Seige, in the "Klinisches Jahrbuch," 1905, Bd. 14, pp. 507-516, reports finding typhoid in the stools of a person one year after having typhoid, and in the stools of another person ten years after having typhoid.

6. Besserer, A., and Jaffe, J., "Deutsche Med. Wochenschr.," 1905, pp. 2044-2047," found typhoid in the feces of the following cases:

Tamble—Patient well—typhoid 4 years before.
No. 52—Patient well—typhoid 7 years before.
No. 2—Patient well—typhoid 3 months before.
No. 25—Same patient as No. 2—typhoid 6 months.
No. 78—Patient well—never had typhoid.

7. Levy, E., and Kayser, H., in the Münch. Med. Wochenschr., No. 50, 1906, report the case of a woman 49 years old who had had typhoid in 1903, and had made a good recovery. In 1905-1906 she was suspected of causing several cases of typhoid, and examination of her feces demonstrated the presence of the bacilli. Later in 1906 the woman died, but not from typhoid, and an autopsy 19 hours after death showed the presence of typhoid bacilli in the spleen, the liver, the bile, in the wall of the gall bladder and inside the gall stones.

8. Dr. P. Klinger, in Arbeiten a. d. Kaiserlichen Gesundheitsamte, Bd. 24, p. 91, reports 23 typhoid carriers in cases under observation during the period from July 1, 1903, to March, 1905. Nine were males and fourteen were females. Eleven of the twenty-three were found while examining the stools of 1,700 well people who had never had typhoid. The other eleven were typhoid convalescents in whom the bacilli had persisted from three months to thirty years. Klinger said that " The plates showed from a single colony to almost a pure culture of typhoid. Sometimes 1 or 2 negative tests would be followed by one showing enormous numbers of typhoid."

9. Kayser, Heinrich, Arbeiten aus dem Kaiserlichen Gesundheitsamte, Vol. 24, p. 176, reports that 13.5 per cent. of the cases of typhoid in Strassburg in 1904-5 were traced to six typhoid carriers. These six typhoid carriers, all women, gave histories of having typhoid. One had had typhoid one year before, one 6 months before, another 10 years, another 24 years, another 27 years, and another 30 years before.

In all the cases the stools and urine were examined. In one case, that of a woman who had had typhoid 27 years before, the bacilli were not found. She was suffering from gall stones, however, and a test of her serum gave a positive Widal reaction.

10. Lentz. " Ueber Chronische Typhus Bacillen-träger, (Klinisches Jahrbuch, xiv., 1905, pp. 475-494), says that seven doctors have reported the following typhoid carriers: the first reports a case of one who had had typhoid three years before, who was known to cause two cases of typhoid; the second reports four typhoid carriers, who had had typhoid as long ago as 42 years, 15 years, 13 years and 12 years, respectively. To these four were traced 12 cases of typhoid. The third

reports a typhoid carrier who had typhoid 19 years before, who caused 6 cases of typhoid. The fourth reports a carrier who had had typhoid 1½ years before, but to whom no cases had been traced; the fifth had a case which had had typhoid 17 years before and had caused 27 cases; the sixth had a case who had had typhoid 10 years before and had caused one other case; and the seventh doctor reports a carrier who had had the disease 17 years before. To this last, two cases were due.

Out of 400 typhoid patients Lentz found that six retained the bacilli from periods ranging from 3 1/3 to 13 months.

When the bacilli were in the stools of typhoid carriers, Lentz found that he could not get rid of them by any treatment. Their retention is due, he thinks, to faulty metabolism and concomitant chronic disease. Faulty care during convalescence may also be a cause. He notes the predominence of women who are carriers, over the men, and especially married women who have borne children. In most cases, the bacilli are present in great numbers. Lentz suggests that the gall bladder may not be the only source, but that the appendix and the deeper folds of the intestines may also be involved.

In conclusion, he suggests the following rules for controlling these carriers:

1. Disinfection of stools.
2. Disinfection of privies.
3. Police notification.
4. Bacteriological examination of stools.
5. Prevention of any occupation where the carrier is in a position to infect others.

11. Goldberger (1907) has an excellent bibliographical and statistical article on "Typhoid Carriers" in Bulletin No. 35 of the Public Health and Marine Hospital Service.

In averaging the results obtained by several observers: Lentz (1905), Klinger (1906), and von Drigalski (1906), he found that of 1,782 cases of typhoid fever, 53, or about 3 per cent., became chronic carriers. The persistence of the typhoid bacilli in the stools is due, at times, he says, "to chronic typhoid ulcerations, but more commonly to the infection of the gall bladder with this bacillus."

12. Soper (1907), Journal of the A. M. A., p. 2019.

REPORT OF THE WEEKLY BACTERIOLOGICAL EXAMINATION OF CROTON WATER FOR THE YEAR 1907.

By W. Carey Noble.

Croton tap water at East Sixteenth street was plated in agar and tested for the presence of colon bacilli once a week during the year. The colony count at 37 degrees and 24 degrees was as follows:

	1 c.c. Plated in Agar.		Quantity of Water Containing Colon Bascilli as shown by the Presumptive Test.
	At 37° C. for 24 hrs.	At 24° C. for 72 hrs.	
January 3	70 colonies.	2,780 colonies.	1 c.c.
January 10	105 "	2,900 "	1 c.c.
January 16	53 "	560 "	10 c.c.
January 24	21 "	183 "	10 c.c.
February 8	30 "	290 "	10 c.c.
February 14	27 "	168 "	10 c.c.
February 20	29 "	240 "	10 c.c.
February 28	13 "	232 "	1 c.c.
March 8	21 "	320 "	10 c.c.
March 15	43 "	520 "	1 c.c.
March 21	72 "	2,422 "	1/10 c.c.
March 28	48 "	2,290 "	1/10 c.c.
April 4	32 "	109 "	10 c c
April 10	57 "	103 "	10 c.c.
April 17	26 "	96 "	10 c.c.
April 24	29 "	102 "	10 c.c.
May 1	13 "	210 "	10 c.c.
May 9	10 "	184 "	10 c.c.
May 15	8 "	83 "	10 c.c.
May 24	18 "	91 "	10 c.c.
May 29	25 "	620 "	10 c.c.
June 5	14 "	710 "	10 c.c.
June 14	20 "	490 "	1 c.c.
June 24	23 "	310 "	1 c.c.
July 3	27 "	107 "	1 c.c.
July 11	19 "	162 "	1/10 c.c.
July 17	16 "	39 "	10 c.c.
July 25	121 "	380 "	1 c.c.

	1 c.c, Plated in Agar.		Quantity of Water containing Colon Bascilli as shown by the Presumptive Test.
	At 37° C. for 24 hrs.	At 24° C. for 72 hrs.	
August 3	28 colonies.	210 colonies.	1/10 c.c.
August 9	86 "	370 "	1/10 c.c.
August 15	11 "	39 "	1/10 c.c.
August 21	18 "	30 "	1 c.c.
August 30	15 "	70 "	1 c.c.
September 5	25 "	37 "	10 c.c.
September 12	.... "	.... "	10 c.c.
September 19	62 "	185 "	10 c.c.
September 25	209 "	420 "	1/10 c.c.
October 2	26 "	65 "	.01 c.c.
October 10	58 "	71 "	1/10 c.c.
October 19	80 "	153 "	1/10 c.c.
October 25	52 "	169 "	1/10 c.c.
October 31	52 "	211 "	1/10 c.c.
November 7	101 "	148 "	1/10 c.c.
November 16	64 "	104 "	1 c.c.
November 25	92 "	270 "	1/10 c.c.
November 30	43 "	115 "	1 c.c.
December 5	56 "	163 "	1 c.c.
December 13	29 "	80 "	10 c.c.
December 18	37 "	103 "	1 c.c.
December 27	52 "	142 "	1 c.c.

THE PRODUCTION OF DIPHTHERIA ANTITOXIN DURING THE YEAR 1907.

By Dr. Edwin J. Banzhaf.

The total amount of antidiphtheric citrated plasma produced in the laboratory during the year 1907 was 599,300 c.c. This amount was recovered from 106 bleedings from 16 horses. The average potency was 390 units per c.c. The highest potency was 850 units per c.c., and the lowest 125 units per c.c.

By the process of refining and concentrating the antitoxic citrated plasma, devised in the laboratory,[1] we produced 193 liters of antitoxic globulin solution with an average potency of 1,000 units per c.c. The highest potency was 1,825 units per c.c., and the lowest 700 units per c.c. Also by a process of fractioning the antitoxic citrated plasma, devised in this laboratory,[2] we produced 62 liters of antitoxic globulin solution with an average potency of 1,250 units per c.c. The highest potency was over 2,000 units per c.c., and the lowest 850 units per c.c.

Horse 262 has produced an enormous quantity of antitoxic serum. This horse entered the antitoxin stable in February, 1904, and soon began to produce a high grade serum. During the year 1904 he produced 81,010 c.c. serum, averaging 496 units per c.c. During the year 1905 he produced 70,085 c.c. serum, averaging 400 units per c.c. During the year 1906 he produced 165,010 c.c., averaging 409 units per c.c. From two bleedings in January, 1907, we recovered 9,800 c.c., with a potency of 450 units per c.c., making the total production from this horse 325,905 c.c., averaging 422 units per c.c.

Under Dr. Parks' direction, horses 318, 319, 320, 322, 323, 324, 325, 326, 327 and 328 were immunized simultaneously against diphtheria and tetanus toxins, each animal subsequently being continued on the toxin to which it responded best. Horses 318 and 320 were the only ones that responded to tetanus toxin, that is to a fairly high potency, whereas horse 320 was refractory to diphtheria toxin.

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[1] Gibson, Journ. of Biol. Chem., Vol. 1, p. 161, and Report of this Department for 1905.

[2] Banzhaf and Gibson, Journ. of Biol. Chem., Vol. iii., p. 253, and Report of this Department for 1906.

After 9 bleedings of horse 318 for tetanus antitoxin, the tetanus immunization was discontinued and the animal was again immunized against diphtheria toxin. After two months' immunization, this horse again produced a fairly high antidiphtheric serum (550 units per c.c.).

The following table shows the production of antitoxin by the individual horses:

Horse Number.	Date of First Injection of Toxin.	Number of Bleedings in 1907.	Citrated Plasma in c.c. produced during 1907.	Highest Potency in Units during 1907.	Average Potency in Units during 1907.
262	2/15/04	2	9,800	450	450
306	2/14/06	2	10,300	300	300
309	9/29/06	2	13,500	200	200
316	10/2/06	2	14,300	525	525
317	11/13/06	1	3,800	200	200
318	1/29/07	19	60,150	600	493
319	1/29/07	13	92,950	600	530
322	2/23/07	5	30,200	625	350
323	3/2/07	22	137,350	850	560
324	3/2/07	2	23,100	125	125
325	3/2/07	9	41,750	300	207
326	3/9/07	5	37,400	600	590
327	3/15/07	2	12,750	400	400
328	3/15/07	1	4,000	550	550
333	6/25/07	10	57,000	400	316
334	7/27/07	9	50,950	500	450

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